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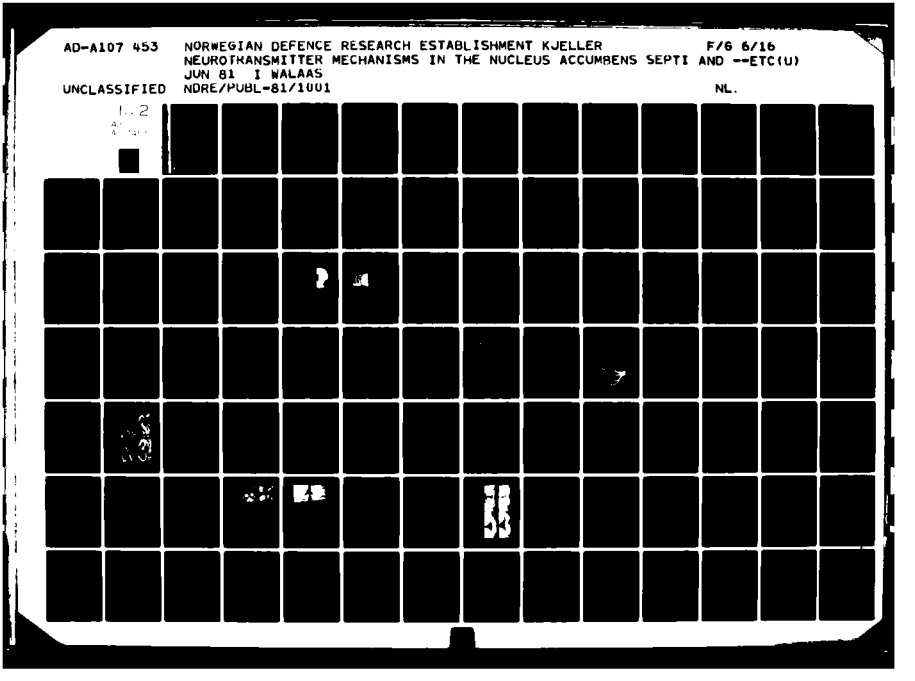

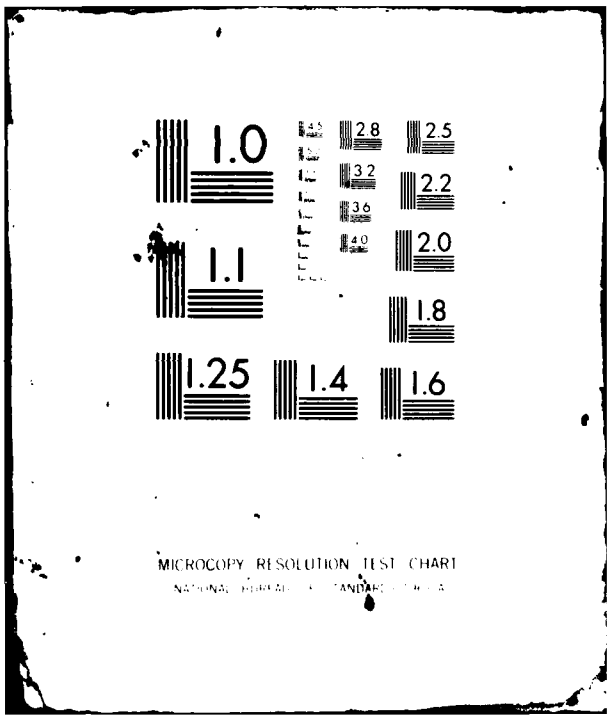
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NEUROTRANSMITTER MECHANISMS IN THE NUCLEUS ACCUMBENS SEPTI AND RELATED REGIONS IN THE RAT BRAIN

BY

IVAR WALAAS

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7) INDEXING TERMS

IN ENGLISH	IN NORWEGIAN
a) γ-aminobutyrate	a) γ-aminobutyrat
b) glutamate	b) glutamat
c) nucleus accumbens	c) nucleus accumbens
d) neostriatum	d) neostriatum
e) hippocampus	e) hippocampus

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8) ABSTRACT (continue on reverse side if necessary)

The present investigation compares the localization of different transmitter candidates, particularly the amino acids γ-aminobutyrate (GABA) and glutamate (GLU), in limbic and basal ganglia regions in the rat brain. In particular, the characteristics of nucleus accumbens septi have been studied in some detail. GABA neurons have been found in nucleus accumbens, and GABA projections from this nucleus have been identified in restricted basal forebrain and mesencephalic regions. GLU projections from the neo- or allocortex have been found to terminate in nucleus accumbens and other forebrain and hypothalamic nuclei. Neurotransmitters in local neurons have been identified in the hippocampus, nucleus accumbens, septum and caudatoputamen by means of local kainic acid injections, while neurons in the mediobasal hypothalamus have been studied after systemic treatment of newborn animals with monosodium glutamate. The results are discussed as a basis for a better understanding of limbic-basal ganglia interactions.

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PREFACE

The present investigation has been carried out at the Norwegian Defence Research Establishment, Division for Toxicology, Kjeller, in the period 1976 – 1979. It represents part of a continuing research program undertaken by the Institute, aimed at the elucidation of biochemical aspects of brain organization and function at the synaptic level. Increasing our knowledge in this area is a prerequisite for understanding drug actions, intoxications and specific disorders of the brain, and for devising rational treatments for these conditions.

I wish to express my gratitude to Dr philos F Fonnum, Head of the Division for Toxicology, for introducing me to the excitement of modern neuroscience, and for his continuing and inspiring support during the investigation. I also wish to thank the Director of the Institute, Mr Finn Lied, for providing research facilities. I am further indebted to Dr R Lund Karlsen for discussions and suggestions during the initial investigations. Mrs K Holte and Mr P J Karlsen have given me technical help, Mrs E Iversen has instructed me in some of the microchemical techniques used, and together with Mrs L Eliassen given me technical assistance during parts of the study. I am also grateful to my colleagues at the NDRE for the stimulating milieu they have provided.

I am particularly grateful to my father and my late mother, who by their own example showed me the attractions of a life in biomedical science, and to my wife for her support throughout the period of this work.

New Haven, Connecticut, June 1981

Ivar Walaas

CONTENTS

NEUROTRANSMITTER MECHANISMS IN THE NUCLEUS ACCUMBENS SEPTI AND RELATED REGIONS IN THE RAT BRAIN

SUMMARY

The present investigation compares the localization of different transmitter candidates, particularly the amino acids γ-aminobutyrate (GABA) and glutamate (GLU), in limbic and basal ganglia regions in the rat brain. In particular, the characteristics of nucleus accumbens septi have been studied in some detail. GABA neurons have been found in nucleus accumbens, and GABA projections from this nucleus have been identified in restricted basal forebrain and mesencephalic regions. GLU projections from the neo- or allocortex have been found to terminate in nucleus accumbens and other forebrain and hypothalamic nuclei. Neurotransmitters in local neurons have been identified in the hippocampus, nucleus accumbens, septum and caudatoputamen by means of local kainic acid injections, while neurons in the mediobasal hypothalamus have been studied after systemic treatment of newborn animals with monosodium glutamate. The results are discussed as a basis for a better understanding of limbic-basal ganglia interactions.

1 OBJECT OF INVESTIGATION

The present study was initiated in order to gain basic information on some aspects of neurotransmitter mechanisms in the mammalian brain. Analysis of regions with dopaminergic innervation seemed of particular importance, due to the suggested role of such regions in movement disorders, endocrinological and mental dysfunction, and antipsychotic drug action (Costa and Gessa 1977, Roberts *et al* 1978). Furthermore, anatomical and functional reclassifications of the limbic system-basal ganglia interface, particularly the nucleus accumbens septi, have recently been proposed (Heimer and Wilson 1975, Heimer 1978, Nauta *et al* 1978, Nauta 1979, Mogenson *et al* 1980). Neurochemical data relevant to these hypotheses have, however, been scarce (Wilson 1972, Nauta 1979). Therefore, the major brain regions chosen for study were related to the 'limbic system' and/or the basal ganglia, and all had a well-defined dopaminergic innervation. During the study, it also became clear that the neurons containing the amino acid transmitter candidates γ-aminobutyrate (GABA) and glutamate (GLU) in these regions were largely unidentified. These transmitter systems have therefore received particular attention.

Initial work analysed the detailed topographical distribution of the monoamine, acetylcholine (ACh) and GABA neurons in the regions by means of enzymatic transmitter marker analysis (Fonnum 1975a) on microdissected tissue samples (Papers I, VI, VIII, XI). The role of the excitatory amino acid GLU was studied by means of amino acid analysis and high affinity GLU uptake activity measurements. In two such studies, the GLU input to nucleus accumbens was identified and compared to that of caudatoputamen (Papers II, III), while two studies analysed allocortical GLU projections from the hippocampal formation to the septum, basal forebrain and hypothalamus (Papers IV, V).

Local transmitter mechanisms were studied by means of 'excitotoxic' amino acid lesions. The hypothalamic toxicity of systemically administered GLU to newborn animals was analysed with morphological, histochemical and microchemical methods

(Paper VI). The toxicity and specificity of local application of the GLU-analogue kainic acid was studied in the hippocampus, a region with well-defined monoamine, ACh and GABA fibers (Paper IV). Later work used this compound in studies on nucleus accumbens, caudatoputamen and septum, where local neurons were identified (Papers II, VII). The cellular and subcellular localization of guanylate cyclase, an enzyme suggested to be involved in ACh and GLU neurotransmission (Greengard 1978, Biggio *et al* 1978) and in kainate neurotoxicity (Honegger and Richelson 1977), was also investigated in the latter nuclei (Paper VII).

Finally, the organization of GABA neurons in the nucleus accumbens was compared to the caudatoputamen, and the participation of such neurons in the accumbens efferents was investigated. Regions in the basal forebrain and mesencephalon receiving accumbens fibers were characterized morphologically, histochemically and biochemically, and compared to regions receiving GABA efferents from the caudatoputamen (Papers I, VIII, IX).

The results have been described in nine separate publications:

I Fonnum F, Walaas I, Iversen E (1977): Localization of GABAergic, cholinergic and aminergic structures in the mesolimbic system. *J Neurochem* 29, 221–230.

II Walaas I, Fonnum F (1979): The effects of surgical and chemical lesions on neurotransmitter candidates in the nucleus accumbens of the rat. *Neuroscience* 4, 209–216.

III Walaas I (1981): Biochemical evidence for overlapping neocortical and allocortical glutamate projections to the nucleus accumbens and rostral caudatoputamen in the rat brain. *Neuroscience* 6, 399–405.

IV Fonnum F, Walaas I (1978): The effect of intrahippocampal kainic acid injections and surgical lesions on neurotransmitters in hippocampus and septum. *J Neurochem* 31, 1173–1181.

V Walaas I, Fonnum F (1980): Biochemical evidence for glutamate as a transmitter in hippocampal efferents to the basal forebrain and hypothalamus in the rat brain. *Neuroscience* 5, 1691–1698.

VI Walaas I, Fonnum F (1978): The effect of parenteral glutamate treatment on the localization of neurotransmitters in the mediobasal hypothalamus. *Brain Res* 153, 549–562.

VII Walaas I (1981): The effects of kainic acid injections on guanylate cyclase activity in the rat caudatoputamen, nucleus accumbens and septum. *J Neurochem* 36, 233–241.

VIII Walaas I, Fonnum F (1979): The distribution and origin of glutamate decarboxylase and choline acetyltransferase in ventral pallidum and other basal forebrain regions. *Brain Res* 177, 325–336.

IX Walaas I, Fonnum F (1980): Biochemical evidence for γ-aminobutyrate containing fibers from the nucleus accumbens to the substantia nigra and ventral tegmental area in the rat. *Neuroscience* 5, 63–72.

Abbreviations used

ACh	– acetylcholine
AChE	– acetylcholinesterase
AAD	– aromatic L-amino acid decarboxylase
ASP	– L-aspartic acid
ChAT	– choline acetyltransferase
GABA	– γ-aminobutyric acid
GAD	– L-glutamate decarboxylase
GLU	– L-glutamic acid

2 GENERAL SUMMARY

The origin, distribution and density of neurons containing different neurotransmitter candidates have been analysed in rat brain regions usually considered to belong to either the 'limbic system' or to the basal ganglia. Particular emphasis has been placed on the neurons connected with the nucleus accumbens septi, and comparisons have been made between the afferent, local and efferent neurons in this region and those in the septum and caudatoputamen (neostriatum). After surgical interruption of allocortical fibers from the hippocampal formation running in the fimbria/fornix bundles, specific decreases were seen in both the activity of the high affinity GLU uptake and in the concentration of endogenous GLU in the lateral septum, the nucleus accumbens, the rostral diagonal band nucleus, the bed nucleus of the stria terminalis, the mediobasal hypothalamus, and the mammillary body. Similar results were also found in the lateral septum after specific chemical destruction of pyramidal cells in the hippocampal formation by means of local kainic acid injection. Thus, most of those allocortical excitatory fibers from the hippocampal formation which run in the fornix bundle appear to use GLU as neurotransmitter.

The origin and distribution of GLU fibers in different parts of the nucleus accumbens and the caudatoputamen were further compared. Fibers from the frontal neocortex were found in all parts of the caudatoputamen and the nucleus accumbens, while fibers from the caudal neocortex were found in the dorsal caudatoputamen only. Allocortical fibers disrupted by fornix lesions were seen in the ipsilateral nucleus accumbens and the ventral caudatoputamen. Thus, the nucleus accumbens and the ventral caudatoputamen are innervated by both neocortical and allocortical GLU fibers, while the dorsal caudatoputamen is reached by neocortical GLU fibers only.

Monoamine fibers were studied by analysis of aromatic L-amino acid decarboxylase (AAD) activity. High density of such fibers was seen in the caudatoputamen, nucleus accumbens and olfactory tubercle, with considerably lower density in the septum. A decrease in AAD activity in all forebrain regions after brain hemitransections confirmed that these fibers are ascending. High AAD activity was also found in the mesencephalic ventral tegmental area and the substantia nigra, pars compacta, which constitute the origins of the major ascending dopaminergic systems, and in the hypothalamic median eminence and arcuate nucleus, regions with a local dopamine neuronal system.

Microinjection of kainic acid into the hippocampus or the nucleus accumbens led to extensive destruction of local neurons without affecting afferent projections. Systemic administration of GLU to newborn animals induced similar local neuronal necrosis in the hypothalamic arcuate nucleus. By these techniques, local cholinergic neurons as measured by choline acetyltransferase (ChAT) activity were found to be highly concentrated in the caudatoputamen, nucleus accumbens and probably also in the olfactory tubercle. Lower density of such neurons was found in the septum, and the latter were insensitive to local kainate injections. In the hypothalamus, ACh neurons, probably originating in the arcuate nucleus, were concentrated in the median eminence. Local GABA neurons, analysed by glutamate decarboxylase (GAD) activity measurements, were highly concentrated in the lateral septum, dorsal olfactory tubercle and nucleus accumbens, with lower density found in the caudatoputamen. The median eminence contained only few GABA terminals, but some of them appeared to belong to a local, tubero-infundibular system.

Kainate injections were also used to analyse the cellular localization of guanylate cyclase, an enzyme suggested to be involved in ACh or GLU transmission. This enzyme was highly concentrated in local neurons in the nucleus accumbens and

caudatoputamen, while the intermediate activity in the septum was insensitive to kainate.

Finally, the descending efferent projections from the nucleus accumbens were analysed. A major GABA projection was found to terminate in the rostral substantia innominata and, less importantly, in the ventral globus pallidus. The characteristics of the substantia innominata supported the designation of this region as a 'ventral pallidum'. A significant GABA pathway was also found to terminate in the rostromedial substantia nigra. These projections further support the "striatal" nature of the nucleus accumbens. In contrast, only a small number of GABA terminals were destroyed in a restricted part of the ventral tegmental area after accumbens lesions. Thus, no major 'feed-back' GABA pathway was identified in the mesolimbic system.

3 DISCUSSION OF METHODS

3.1 Dissection of samples

The present study has tried to identify neurotransmitter mechanisms by either correlating the distribution of neurochemical markers with the distribution of specific neuronal populations, or by following the changes in these markers induced by degeneration of different neurons (Fonnum 1975a, Storm-Mathisen 1977a). In principle, sufficiently sensitive assay methods should allow neurochemical studies on very small populations of neurons. Histochemical, particularly immunocytochemical methods are presently the most sensitive and specific tools for such studies (Hökfelt *et al* 1980). However, these methods are not generally available for the study of small-molecular weight compounds, and they also do not give quantitative results. This study has therefore used chemical analysis of microdissected samples, following anatomical descriptions of the neuronal populations. In detailed distribution studies of small regions, microdissection on freeze-dried sections was employed (Lowry 1953). This approach allows analysis of submicrogram samples (*e g* Paper IX), but precludes study of labile activities such as uptake mechanism. However, it allows simultaneous inspection of the experimental sample and morphological controls in the dissection microscope, and facilitates later dissection of fresh tissue slices or microwave-inactivated brain tissue. Furthermore, the present work has consistently studied samples with 'normal' anatomical outlines, in contrast to the widely used 'punching' technique (Palkovits 1973). The advantage of this approach was particularly evident in the hypothalamus and mesencephalon (Papers I, VII, IX).

3.2 Lesioning techniques

Most nerve fiber projections in the brain terminate in a very precise and restricted pattern The small, restricted lesions used by anatomists to induce anterograde degeneration therefore usually do not lead to measurable chemical changes in the samples analysed by neurochemists, except in certain cases, *e g* the septohippocampal and striatonigral projections (Fonnum *et al* 1974, Lewis *et al* 1967). Surgical interruption of projections must therefore include most of the fibers under study (Storm-Mathisen 1977a), which introduces problems of specificity. These lesions, whether electrolytic or crude transections, will usually encroach upon other regions, destroy fibers 'en passage' and possibly damage the blood supply to different regions. I have approached these problems both by making crude lesions, *e g* brain hemitransections, and analysing chemical changes in restricted regions, and by making more restricted lesions and analysing both the target regions and adjacent nuclei. Most of the conclusions drawn in this work are based on a combination of these approaches. Morphological controls

of the surgical lesions were also carried out, either by staining sections (Paper VIII, IX), or by inspecting the brains during the sample preparation. Other chemical activities were also analysed in or near the target areas, to exclude unspecific effects.

Surgical lesions are unable to identify the local neurons present in most brain regions, even if retrograde changes are sometimes observed (*e g* Quik *et al* 1979). Specific neurotoxins have been described for monoamine neurons, *e g* 6-hydroxydopamine (Ungerstedt 1971), but other specific toxins have not been described. A major advance in neurobiology was therefore made when the lesioning properties of certain acidic amino acids were discovered. Initial observations (Lucas and Newhouse 1957) demonstrated that GLU when systemically applied to newborn rodents was toxic to certain neurons in the retina. The GLU effects were further explored in detail by Olney and coworkers (Olney 1974, 1979), who showed that most or all local neurons were destroyed in those regions where GLU penetrated the blood-brain barrier, *i e* the hypothalamic median eminence and arcuate nucleus. Afferent fibers or glial cells were not destroyed. The potent GLU-analogue kainic acid (Olney *et al* 1974) was therefore tested in the caudatoputamen, and similar results were seen, *i e* a destruction of local neurons, no major effects on afferent fibers, and a reactive gliosis (Coyle and Schwarcz 1976, McGeer and McGeer 1976, 1978, Schwarcz and Coyle 1977). The mechanism of action of this compound remains undefined (Nadler 1979), the effect seems to be linked to intact excitatory inputs (McGeer *et al* 1978) which however may use different transmitters (Nadler 1981). Guanosine 3′,5′-monophosphate (cyclic GMP) has also been suggested as an intermediary in the effect (Honegger and Richelson 1977).

We extended the results from caudatoputamen to the hippocampus, chosen because of its well-defined transmitter chemistry (Storm-Mathisen 1977a) which is very different from that of the basal ganglia regions (Paper IV). After infusion of high doses of kainate into the hippocampus we observed similar results, *i e* no effect on neurochemical or histochemical markers for afferent fibers, but substantial decreases in markers for local neurons. Similar results were reported simultaneously (Schwarcz *et al* 1978). Morphological controls indicated little or no damage to other brain regions (Paper IV). This was surprising, as later studies have shown that kainate, especially when injected into the hippocampus, usually induces major necrotic changes in both adjacent and remote brain regions (Wurthele *et al* 1978, Zaczek *et al* 1980). Thus, both the subiculum, entorhinal cortex and contralateral hippocampus have been reported to be affected by this treatment, in contrast to our results. Similarly, the piriform cortex is often destroyed by intrastriatal kainate (Zaczek *et al* 1980), while my injections of kainate into the nucleus accumbens usually left this cortical region untouched (Paper II). The reasons for these more specific lesions are not clear, but are probably a result of my use of diazepam in the anesthetic used for lesioning the animals. Thus, both benzodiazepines and long-acting barbiturates have been reported to prevent the remote effects of kainate without changing the local toxicity (Zaczek *et al* 1978, Ben-Ari *et al* 1979).

In conclusion, the studies of kainate effects in nucleus accumbens, septum and caudatoputamen (Papers II, VI) are probably valid as identifications of local transmitter mechanisms. However, the effects on very short-axoned projections (*e g from the bed* nucleus of the stria terminalis to the nucleus accumbens (Swanson and Cowan 1979) cannot be ascertained from these studies.

3.3 Biochemical methods

The identification of neurotransmitters relies on the fulfilment of the criteria of presence, release and identity of action (Werman 1966, Storm-Mathisen 1977a). Of these, the presynaptic criteria (presence, release) are crucial for the biochemical identification of a transmitter system, while the postsynaptic action is electro-physiologically defined (Werman 1966). Biochemical studies on receptor-binding or second messenger generation are not conclusive as these activities may be present outside the relevant synapses (Storm-Mathisen 1977a). The present study has, however, used both pre- and postsynaptic activities to study the characteristics of the regions. The presynaptic markers include enzyme activities, high affinity uptake systems and analysis of endogenous amino acids.

3.3.1 Determination of enzyme activities

ChAT is known to be a specific marker for cholinergic neurons, while AChE is not (Fonnum 1975a). The methods used for these enzymes have been developed in this laboratory (Fonnum 1969, 1975b), and are known as rapid, simple and extremely sensitive, suitable for assays of submicrogram samples (Fonnum 1970, Storm-Mathisen 1970). Triton X-100 has always been included in these assays, to release occluded activity (Fonnum 1966).

GAD is also a specific marker, in this case for GABA neurons, with only very low activity found outside these neurons (Fonnum and Storm-Mathisen 1978). GAD was analysed with a CO_2-trapping micromethod (Fonnum *et al* 1970). The possibility of non-GAD CO_2 production from GLU was controlled by inclusion of Triton X-100 in the incubation medium (MacDonnell and Greengard 1975).

AAD is concentrated in both dopamine, noradrenaline and serotonin neurons, but is also present in non-neuronal structures (Fonnum 1975a, Kellogg *et al* 1973). It is an acceptable marker for monoamines in regions with high activity, *e g*, substantia nigra, caudatoputamen, while results from regions with low enzyme activity must be evaluated more carefully. The analytical technique used was based on liquid cation exchange (Broch and Fonnum 1972), similar to the ChAT method.

Carnitine acetyltransferase is known as a mitochondrial enzyme (McCaman *et al* 1966), and was analysed both as a subcellular marker (Paper II) and as a general tissue marker (Paper VI) with a liquid cation-exchange technique (Sterri and Fonnum 1980). *5'-nucleotidase* is known as a membrane marker, possibly concentrated in glial cells (Kreutzberg *et al* 1978), and was assayed by a sensitive radioenzymatic method (Avruch and Wallach 1971).

Guanylate cyclase was analysed by a radioenzymatic method based on that described by Krishna and Krishnan (1975). Due to the controversy described in Paper VI, this activity was characterized in more detail by the use of activators and extraction procedures, and by identification of the product by chromatography and by treatment with phosphodiesterase (Paper VI). Some experiments were also done with [^{3}H]-GTP as substrate and an alumina-anion exchange purification technique (Mao and Guidotti 1974). This method was less sensitive, but gave the same subcellular and topographical distribution as that found with the method based on [α-^{32}P] GTP (unpublished). The specific activities found in the soluble fractions were higher than those reported by other workers (Hofmann *et al* 1977). The reason for this is unknown, but large variations in brain guanylate cyclase activity have previously been presented in separate publications by the same investigator (Bartfai *et al* 1978, Tjörnhammar *et al* 1979). The enzyme is therefore probably sensitive to a number of unidentified factors.

3.3.2 Acidic amino acids

The lack of specific, sensitive markers for the excitatory amino acid transmitter candidates was a major obstacle in the investigation of the role these compounds played in the brain (review, Davidson 1976). The demonstration of a sodium-dependent, high affinity uptake of GLU and aspartate (ASP) into nerve terminals (Logan and Snyder 1971) was a major step forward. However, the lack of discrimination between GLU and ASP, and between other similar compunds (Balcar and Johnston 1972), made it clear that additional parameters would have to be studied. A seminal paper from Snyder and coworkers (Young *et al* 1974) indicated that analysis of free amino acids might be combined with uptake analysis in studies on putative GLU or ASP fibers. Later studies supported this strongly (Divac *et al* 1977, McGeer *et al* 1977a, Kim *et al* 1977, Storm-Mathisen 1977b). This approach was therefore adopted (review, Fonnum *et al*, 1979, see, however Cotman *et al* 1981).

Amino acid analysis was initially done (Papers II, IV, VII) with a double isotope dansyl-chloride technique (Karlsen and Fonnum 1976). The samples were dissected from animals killed by focussed microwave irradiation of the brain, a technique developed to minimize post-mortem changes in labile compunds (Guidotti *et al* 1974). Such changes are known to afflict GABA (Alderman and Shellenberger 1974, Balcom *et al* 1975), but not GLU (Balcom *et al* 1976). However, the mediobasal hypothalamus and basal forebrain were difficult to dissect after this procedure, and rapid cooling and freezedrying of the samples were adopted for a later study (Paper V), in which an automic amino acid analyser was used for quantitation. Results from nucleus accumbens and septum were comparable when analysed with either of the two methods (Papers II, IV, V).

High affinity uptake mechanisms were studied in sucrose homogenates containing nerve terminals (Fonnum *et al* 1979). Experiments showed that the accumulation of [^{3}H] L-GLU in this preparation occured into synaptosomes, and was linear with amount of tissue (Paper II). Thus, further purification of the nerve terminals (*e g* McGeer *et al*, 1977a, Zaczek *et al*, 1979) was deemed unnecessary. Lesion-induced changes in this uptake have been shown to represent changes in the number of uptake sites and not in the affinity for the substrate, in a number of systems (Young *et al* 1974, Storm-Mathisen 1978, Zaczek *et al* 1979). The question of homo-exchange of labelled GLU with the intracellular GLU (Levi and Raiteri 1974) is not important in this context, as my studies were aimed only at studying the number of uptake sites present. However, the possibility of different putative GLU nerve terminals having different affinity of GLU, as has been found for GABA (Storm-Mathisen 1975) and suggested for GLU in hippocampus (Storm-Mathisen 1978), is important. Thus, the regional distribution of the uptake may not necessarily indicate the true density of GLU or ASP terminals (see, *e g* Paper III). Also, the GLU uptake into glial cells (Henn *et al* 1974) may have a regional variation (Schousboe and Divac 1979). However, this glial uptake does not appear to interfere significantly in my uptake conditions (Fonnum *et al* 1979).

4 GENERAL DISCUSSION

4.1 Characteristics of the nucleus accumbens

Most early workers related the nucleus accumbens to the basal ganglia (Ariens Kappers and Theunissen 1907), particularly as a part of the "olfactostriatum" (Herrick 1926). The exact relationships of the region remained unknown, however, until inputs from the hippocampal formation and stria terminalis were demonstrated (Fox 1943, Raisman *et al* 1966, de Olmos and Ingram 1972). These results linked the nucleus closely with the "limbic system". The cytoarchitectonics of the nucleus were

described in different species (*e g* from mouse to elephant) (Koikegami *et al* 1967), and reported to be similar to those of the caudatoputamen (Wilson 1972). The nucleus began to attract major interest, however, only after the dense dopaminergic innervation of the nucleus, originating in the socalled A10 cells in the mesencephalic ventral tegmental area was reported (Andén *et al* 1966, Ungerstedt 1971). The simultaneously developed "dopamine theory of schizophrenia", *i e* that psychotic symptoms in amphetamine-induced psychosis and possibly in schizophrenia occured together with hyperactivity in dopaminergic neurotransmission (Snyder 1973, Hökfelt *et al* 1974), led to a great interest in the so-called mesolimbic dopamine systems, particularly that of the A10-nucleus accumbens fibers (reviews, Costa and Gessa 1977). Other anatomical characteristics were soon described, particularly the efferent projections to the substantia innominata (Wilson 1972, Williams *et al* 1977, Nauta *et al* 1978), and to the globus pallidus, the ventral tegmental area and the substantia nigra (Swanson and Cowan 1975, Conrad and Pfaff 1976, Nauta *et al* 1978). The nucleus was therefore reclassified (Heimer and Wilson 1975) as being a part of the "ventral striatum", innervated by the limbic cortex but projecting most densely to basal ganglia-related regions (Heimer 1978).

4.2 Neurochemical characteristics

4.2.1 Introduction

When this study was initiated, several neuronal links had been chemically defined in the basal ganglia (reviews, Brownstein 1979, Fonnum and Walaas 1979). Initial work was therefore aimed at a comparison between the nucleus accumbens, the olfactory tubercle, the septum and the caudatoputamen. Striking similarities were seen in the densities of the monoamine, ACh and GABA systems in the nucleus accumbens and olfactory tubercle when compared with the caudatoputamen. No evidence for a descending GABA projection from these regions to the A10-cells was found, however, in contrast to the major striatonigral GABA tract (Paper I). Thus, biochemical differences appeared to exist between the region.

4.2.2 Cortical fibers

Preliminary reports indicating that GLU could be transmitter of the excitatory corticostriatal (Divac *et al* 1977, McGeer *et al* 1977a, Kim *et al* 1977) and hippocamposeptal fibers (Storm-Mathisen and Opsahl 1978) led to further studies on nucleus accumbens and septum. Active high affinity GLU uptake was found in both regions, and a detailed study in nucleus accumbens showed that this activity appeared to be localized in certain nerve terminals (Paper II). Further, lesions which involved the fornix were able to decrease the activity in the ipsilateral nucleus accumbens. Similarly, fornix transections and destructions of hippocampal neurons with kainic acid decreased the GLU uptake in the lateral septum, while the medial septum was unaffected (Paper IV). Amino acid analysis after these lesions showed that GLU was the only free amino acid which decreased in concentration after the lesion (Papers II, IV, V). Thus, the reductions in GLU uptake were apparently a result of degeneration of GLU, as opposed to ASP terminals (Fonnum *et al* 1979).

These results all conformed to the anatomical studies which were published while this work was in progress (Swanson and Cowan 1977, Meibach and Siegel 1977). Thus, the fibers to nucleus accumbens were shown to arise in the subicular part of the hippocampal formation and to travel through the fimbria to the ipsilateral nucleus accumbens, while most of the fibers to the lateral septum arose in the hippocampus proper and terminated on both sides of the lateral, but not the medial septum. Further, all these fibers were reported to be excitatory (McLennan and Miller 1974, DeFrance and Yoshihara 1975).

The suggestion that GLU might be the major transmitter in the allocortical fibers to the nucleus accumbens and septum has later been supported by other workers which have employed both similar surgical/chemical lesions (Zaczek *et al* 1979, Wood *et al* 1979) and total hippocampal extirpations (Nitsch *et al* 1979). Therefore, the high affinity GLU uptake was used in a study of the distribution of different corticofugal fibers in the rostral caudatoputamen and nucleus accumbens (Paper III). Surprisingly, also the ventral caudatoputamen had some GLU-accumulating terminals destroyed by a fornixlesion, and only the dorsal caudatoputamen appeared to be connected only with the neocortex. Similarly, the nucleus accumbens was found to be innervated from the ipsilateral frontal cortex. Anatomical studies support the latter findings (Beckstead 1979), while the proposed fornix fibers to the ventral caudatoputamen are unconfirmed (Swanson and Cowan 1977).

The combined amino acid and GLU uptake analysis was also used in a study on other fibers in the fornix bundle to the basal forebrain and hypothalamus (Paper V). The previously suggested GLU fibers to the mammillary body (Storm-Mathisen and Opsahl 1978, Nitsch *et al* 1979) were confirmed. In addition, inputs to the anterior diagonal band nucleus, the bed nucleus of the stria terminalis and the mediobasal hypothalamus, all probably arising in the subiculum (Chronister *et al* 1976, Swanson and Cowan 1977) were identified as possible GLU projections (Paper V). However, the lesions also encroached upon the stria terminalis, and the possible contribution of this fiber bundle to the observed decrease in GLU uptake and concentration in the stria terminalis nucleus and mediobasal hypothalamus (de Olmos and Ingram 1972) remains to be defined. It is, however, clear that the GLU system in the hypothalamus is extrinsic, as a hypothalamic deafferentation has been reported to decrease the GLU concentration in hypothalamus considerably (Racagni *et al* 1981).

4.2.3 Local neurons in GLU-innervated regions

The demonstration of a specific neurotoxic action of kainic and glutamic acid, which were suggested to destroy only neurons intrinsic to the regions treated with the neurotoxins (Coyle and Schwarcz 1976, McGeer and McGeer 1976), led to a study of the effects of kainic acid in the hippocampus (Paper IV). The results supported the claims for specificity and toxicity of kainate actions. This neurotoxin was therefore used in the nucleus accumbens, septum and caudatoputamen (Paper II, VI) and the results compared to previous reports from the caudatoputamen (Schwarcz and Coyle 1977, McGeer and McGeer 1978). Most, and probably all cholinergic terminals in the nucleus accumbens and caudatoputamen were found to belong to local, kainate-sensitive neurons, while the moderate amount of such fibers in the septum probably arrives partly from the diagonal band (Emson 1978) and partly belong to local, kainate-insensitive cells in the medial septum (Malthe-Sørenssen *et al* 1980). Thus, the cholinergic system in nucleus accumbens and caudatoputamen was clearly different from that in the septum.

In contrast, all regions had apparently only local GABA cells. These were most concentrated in the lateral septum, dorsal olfactory tubercle and nucleus accumbens (Papers I, IV), and most of them disapperared in all regions after local kainate injections (Papers II, VI). Thus, intrinsic GABA neurons are present in both the nucleus accumbens, septum and caudatoputamen.

A decrease in the GLU uptake in nucleus accumbens was also observed after local kainate injection (Paper II). Thus, some intrinsic GLU-cells may exist in this region. However, it appears more likely that the kainate-sensitive GLU fibers in the nucleus are derived from neighbouring cortical regions destroyed by kainate (Wurthele *et al* 1978, Zaczek *et al* 1980).

A functional link between cholinergic or GLU neurotransmission and cyclic GMP synthesis has been proposed from studies in both the peripheral and central nervous systems (review, Greengard 1978, Ferrendelli 1978). The distribution of guanylate cyclase was therefore also analysed (Paper VI). The enzyme was found in both particulate and soluble fractions in both the nucleus accumbens, caudatoputamen and septum, with particularly intense soluble activity in the nucleus accumbens and caudatoputamen. The particulate enzyme was evenly distributed in all regions. Contrary to the results of other workers (Tjörnhammar *et al*, 1979) both enzyme forms were kainate-sensitive in the caudatoputamen and nucleus accumbens, but not in the septum. Thus, this "second messenger" system also emphasizes the similarities between the nucleus accumbens and the caudatoputamen.

The mediobasal hypothalamus has also a well-defined dopamine system, with cell bodies located in the arcuate nucleus and terminals in the median eminence and pituitary (Lindvall and Björklund 1978). The organization of ACh and GABA neurons is less well known, however (Brownstein *et al* 1976). As nucleus arcuatus and its fibers to the median eminence are the major targets of systemically administered GLU (Olney 1979), the neurochemical results of this lesions were analysed (Paper VII). Initial studies identified a cholinergic system, probably originating in the arcuate nucleus and terminating in the median eminence (Karlsen *et al* 1977) similarly to the results of Carson *et al* (1977). AChE-staining was also seen on arcuate neurons and in the median eminence, findings which also were confirmed by Carson *et al* (1978). In our material, the stain disappeared in the GLU-lesioned animals. This was also observed by others in the arcuate nucleus, but apparently not in the median eminence (Carson *et al*, 1978).

Similarly, a decrease in GAD was seen in the median eminence after GLU-treatment, indicating either a small tuberoinfundibular GABA system from the arcuate nucleus, or some intrinsic GABA cells in the median eminence. GABA terminals have later been demonstrated in the median eminence by autoradiography (Tappaz *et als, 1980),* and the decrease in GAD following GLU treatment has been confirmed by some (Tappaz *et al*, 1981) but not by other workers (Nemeroff *et al* 1977). The dissection procedure seems crucial in this respect (Paper VII). In conclusion, GABA terminals are probably present in the hypothalamic-pituitary interface, where they may play important functional roles (Racagni *et al*, 1981, Olney and Price 1981).

In contrast to the ACh and GABA markers, no effects were seen in the markers used for the local dopamine cells, *i e* AAD activity and high affinity uptake of dopamine. These results are not conclusive, however, as AAD is an unspecific marker (section 3.3.1), and the uptake studies were done on more crudely dissected samples. Also, a recent study has questioned the very existence of high affinity dopamine uptake in the median eminence (Demarest and Moore 1979), even if this activity had been used with apparent success as a marker by others in this region (Cuello *et al* 1973). Thus, the question of dopamine neurons and GLU toxicity in the arcuate nucleus should be reinvestigated with more specific methods. Indeed, other workers have reported a decrease in tyrosine hydroxylase in the arcuate nucleus and median eminence after administration of very high GLU doses (Nemeroff *et al* 1977).

4.3 Nucleus accumbens efferents

4.3.1 Projections to the basal forebrain

The major efferent projection of the nucleus accumbens was first identified by Wilson (1972) to terminate in the rostral substantia innominate. The characteristics and even the outline of this region were until then largely unknown, but it was soon character-

ized as a 'ventral pallidum' (Heimer and Wilson 1975, Heimer 1978). Simultaneous studies by us (Paper I) showed that this region had the highest density of GABA terminals in the rat brain. In a more detailed study (Paper VIII), more than 50% of these GABA fibers were found to be destroyed after accumbens destructions. A less impressive GABA projection was also found to terminate in the anteroventral globus pallidus. Our results thus confirmed the detailed anatomical mapping of the accumbens efferents published by Nauta *et al* (1978), and the physiological studies of Dray and Oakley (1978) and Jones and Mogenson (1980a).The importance of these GABA projections has not been defined, but they appear to be involved in locomotion (Pycock and Horton 1976, Jones and Mogenson 1980b, Mogenson *et al* 1980).

Our results thus supported the concept of a 'ventral pallidum' represented by the rostral part of the substantia innominata in the rat brain (Swanson 1976). This region can apparently be histochemically defined by iron staining with Perl's method (Hill and Switzer 1979). By this method, the region is seen to extend rostrally as fingerlike extensions, each of which is 'capped' by one of the insulae Callejae (Hill and Switzer 1979). Our results appear to support this, as we also found high GAD activity and low AChE staining in the region rostral to the commissura anterior and situated between the nucleus accumbens and the olfactory tubercle (Papers I, VIII). These results on GAD were also confirmed and extended by Okada *et al* (1977).

4.3.2 Projections to the mesencephalon

The caudatoputamen is known to send a major projection to the substantia nigra (Grofova' and Rinvik 1970). These fibers were early demonstrated to be inhibitory (Yoshida and Precht 1971, Precht and Yoshida 1971), probably GABAergic (Kim *et al* 1971, Fonnum *et al* 1974), and surmised to represent feed-back fibers responsible for regulating the activity of the nigrostriatal dopamine neurons (Anden *et al* 1964, Anden and Stock 1973, Cattabeni *et al* 1979, Bunney and Aghajanian 1978). The possibility of a similar 'mesolimbic' feed-back system therefore attracted great interest as a possible link in the neuronal system which was believed to be involved in psychotic phenomena (Stevens 1979). We therefore compared the A10 region with the substantia nigra, and found that the major difference consisted of a low density of GABA terminals in the A10 region, while the substantia nigra, pars reticulata displayed a very dense population of such fibers (Paper I). Furthermore, no evidence for a GABA projection from the forebrain to the A10 region could be found after lesions which both transected the striatonigral GABA fibers in the capsula interna and the ascending monoamine fibers in the medial forebrain bundle. Similar results were reported by McGeer *et al* (1977b). Thus, the transmitter of the accumbens-mesencephalic projection (Swanson and Cowan 1975, Conrad and Pfaff 1976) remained unknown.

However, later reports stated that most of the striatonigral GABA fibers probably did not represent feed-back fibers, and that they rather might constitute an output system (Arbuthnott 1978, Scheel-Krüger *et al* 1977, Di Chiara *et al* 1979) which probably terminated in the pars reticulata on interneurons and output neurons projecting to the tectum, thalamus or reticular substance (Domesick 1977. Nauta and Domesick 1979, Grace and Bunney 1979, Waszczak *et al*, 1980). Nauta and coworkers (Nauta *et al* 1978) demonstrated that this also might be the case for the accumbens projection, which terminated most densely in the substantia nigra, not in the A10 region. Usunoff *et al* (1979) confirmed this at the electron microscopic level. We therefore reinvestigated the distribution of GAD in the mesencephalon after electrolytic destructions of the nucleus accumbens (Paper IX), and found that a considerable number of GABA terminals had disappeared in the rostromedial substantia nigra after this lesion. About half of the nigral GABA terminals in this restricted region appeared to originate in the nucleus accumbens. However, the GABA fibers in the rest of the

substantia nigra were unaffected, which explains the previously negative findings of Dray and Oakley (1978).

Thus, a descending accumbens GABA projection terminates in a nigral region with few or no dopamine cells projecting back to the nucleus accumbens (Nauta *et al* 1978). This GABA projection might therefore be a part of the striatonigral GABA output pathway (Paper IX). The question of a feed-back projection to the dopamine neurons was still unsolved, however, as both physiological and neurochemical studies had presented evidence for such a pathway to the A10-cells (Wolf *et al* 1978, Waddington and Cross 1978). Our new results again demonstrated much lower density of GABA terminals in the A10 region than in the substantia nigra. Furthermore, no change was seen in GAD in the caudal A10 after accumbens lesions (Paper IX), and only a small decrease in GAD was seen in the rostral A10. The demonstration of a GABA-mediated inhibition from nucleus accumbens to some A10 dopamine cells which projected back to nucleus accumbens was therefore surprising (Yim and Mogenson 1980). The A10 region has recently been shown to be more complex than originally believed, with a multitude of different inputs converging on the local cells (Phillipson 1979). My studies of the region, performed with precise microdissection and extensive morphological controls (Papers I, IX), do not exclude that some of these inputs could be GABAergic, but indicate that such projections must be quantitatively very small compared to the striatonigral and accumbens nigral "output" GABA pathways.

Other neuronal systems not analysed in the present study are also present in the basal ganglia-mesencephalic circuits, and some will be briefly mentioned. Substance P-fibers are present in the striatonigral projection (Brownstein *et al* 1977, Jessell *et al* 1978), and these fibers do apparently terminate on both dopamine and non-dopamine cells (Michelot *et al* 1979). No such fibers have been seen in the accumbens efferents (Kanazawa *et al* 1980). Substance P in the A10 region is derived from the habenula, not from the basal ganglia (Cuello *et al* 1978). The distribution of the cholinergic system (Paper I) is in general agreement with other workers, *i e* extremely dense in the interpeduncular nucleus, and very sparse in the substantia nigra (Kataoka *et al* 1973, Fonnum *et al* 1974). No change was observed in this transmitter after rostral hemitransections, in contrast to a recent study which suggested that part of ChAT in the interpeduncular nucleus derives from the diagonal band region (Gottesfeld and Jacobowitz 1978). However, the bilateral termination of the fasciculus retroflexus in the interpeduncular nucleus (Herkenham and Nauta 1979) may have obscured small changes. Also, some of our lesions may have spared the initial, ventral part of the stria medullaris which convey the fibers from the diagonal band (Swanson and Cowan 1979). However, the results in Paper I still indicate that most of the ACh fibers in the interpeduncular nucleus originate in regions caudal to the globus pallidus, *e g*, in the habenula (Kataoka *et al* 1973, Cuello *et al* 1978).

In conclusion, the present study has demonstrated that the major cortical input to the limbic forebrain nuclei, among them the nucleus accumbens, appears to be GLU fibers coming through the fornix similar to the innervation of other limbic and hypothalamic nuclei. However, the nucleus accumbens also shares a neocortical GLU projection with the caudatoputamen. The local transmitter systems and neurochemical characteristics of nucleus accumbens are strikingly similar to those in the caudatoputamen, and the major GABA efferent are also organized similar to, but not identical with, the output from the caudatoputamen. Thus, this study supports the suggested dual limbic-basal ganglia role of the nucleus accumbens.

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LOCALIZATION OF GABAERGIC, CHOLINERGIC AND AMINERGIC STRUCTURES IN THE MESOLIMBIC SYSTEM

F. FONNUM, I. WALAAS and E. IVERSEN
Norwegian Defence Research Establishment, Division for Toxicology, N-2007 Kjeller, Norway

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Abstract—The localization of cholinergic, GABAergic and aminergic structures in the 'mesolimbic' system has been discussed from studies on the topographical distribution of choline acetyltransferase, glutamate decarboxylase and aromatic amino acid decarboxylase in normal rat brain and in brains hemitransected at the level of globus pallidus. The structures analysed included nucleus accumbens, olfactory tubercle, septum, medial forebrain bundle, striatum, substantia nigra, ventral tegmental area and nucleus interpeduncularis.

Choline acetyltranferase was highly concentrated in the nucleus interpeduncularis, but it did also exhibit considerable activity in the nucleus accumbens, the olfactory tubercle and the striatum. The activities did not change after hemitransection. Aromatic amino acid decarboxylase was highly concentrated in the ventral tegmental area, but high activities were also found in the striatum, the nucleus accumbens, the olfactory tubercle and the pars compacta of the substantia nigra. The activity decreased in all areas rostral to the hemitransection. Glutamate decarboxylase was highly concentrated in the dopamine innervated regions, moreso in the limbic structures than in the striatum. Much higher activity was found in the substantia nigra than in the ventral tegmental area. After hemitransection the activity in the substantia nigra was decreased whereas in the ventral tegmental area it was unchanged. Our results thus suggest that dopaminergic cells in the ventral tegmental area do not receive GABAergic fibres from the terminal regions of the ascending dopaminergic fibres. In addition, we found a very high concentration of glutamate decarboxylase in a region traversed by the rostral medial forebrain bundle. Here the activity was mainly confined to the particulate fraction, probably the synaptosomes. This fraction also displayed a very active high affinity uptake of γ-aminobutyric acid.

IT HAS been shown that two of the major dopaminergic fibre systems in mammalian brain, the nigrostriatal and the 'mesolimbic', display several strikingly similar features. They both originate in the mesencephalon, where the fibres to striatum arise from the pars compacta of the substantia nigra (the socalled A9 cell group), and the fibres to the nucleus accumbens, the olfactory tubercle and the septum arise from the ventral tegmental area of Tsai, which corresponds to the A10 cell group (ANDÉN *et al.*, 1964; DAHLSTRÖM & FUXE, 1964; UNGERSTEDT, 1971; LINDVALL & BJÖRKLUND, 1974; SIMON *et al.*, 1976). The neostriatum and the above mentioned limbic regions have similar composition of transmitter candidates. They contain a high level of acetylcholinesterase (AChE, EC 3.1.1.7) (JACOBOWITZ & PALKOVITS, 1974), choline acetyltransferase (ChAT, EC 2.3.1.6) (PALKOVITS *et al.*, 1974; KATAOKA *et al.*, 1975), and furthermore the dopamine receptors in striatum and nucleus accumbens behave similarly with respect to synthesis of cyclic AMP (CLEMENT-CORMIER *et al.*, 1974). Neurophysiological studies indicate that both dopamine and γ-aminobutyric acid (GABA) are inhibitory transmitters in these regions (WOODRUFF *et al.*, 1976). Finally, also the efferent connections of the above-mentioned regions show some similarities. Thus the inhibitory GABAergic fibres which terminate in the substantia nigra arise in the nucleus caudatus putamen and/or globus pallidus (FONNUM *et al.*, 1974; HATTORI *et al.*, 1973; KIM *et al.*, 1971; PRECHT & YOSHIDA, 1971), while the nucleus accumbens projects to the ventral tegmental area and the substantia nigra (CONRAD & PFAFF, 1976; SWANSON & COWAN, 1975). The transmitter in this pathway, however, remains unknown.

The aim of the present investigation was to compare the localization of neurotransmitters in the substantia nigra, in the neostriatum and in some 'mesolimbic' structures, e.g. the nucleus accumbens, the olfactory tubercle, the septum, the nucleus interpeduncularis, and the ventral tegmental area. The topographical distribution of glutamate decarboxylase (GAD, EC 4.1.1.15) and ChAT in these regions was therefore studied in microdissected freeze-dried samples from normal brains, and from unoperated and operated sides of rat brains which had been hemitransected at the level of the globus pallidus. As the distribution of the ascending aminergic fibres are strictly ipsilateral (LINDVALL & BJÖRKLUND, 1974; UNGERSTEDT, 1971), aromatic amino acid decarboxylase (AAD, EC 4.1.1.26) was studied in operated brains using the unoperated side as control. These

studies allowed a more detailed mapping of the distribution than those reported by other workers (KATAOKA *et al.*, 1975; PALKOVITS *et al.*, 1974; TAPPAZ *et al.*, 1976). In the operated brains, a decrease in enzyme activities was taken as evidence of degeneration of ascending or descending fibres containing the respective transmitters. During this study, we made the interesting observation that parts of the region traversed by the rostral medial forebrain bundle (MFB) contained a very high level of GAD which merited further investigation. Part of this work has been presented in a preliminary form (FONNUM *et al.*, in press (*a*)).

MATERIALS AND METHODS

Surgical operation. Albino rats of both sexes weighing 150–250 g were anaesthetised by administration i.p. with Nembutal Vet (Abbott) or Hypnorm Vet (Janssen Pharm. Comp.). A transverse cut was made in the left part of the skull 0.5–1 mm in front of the bregma, the dura mater was split with a scalpel and a hemitransection of the brain was performed with a blunt spatula placed at 90° to the skull surface. The transection went from the midline and 4–5 mm laterally, and in most cases passed through the brain at the level of the rostral part of the globus pallidus, thus interrupting all ascending and descending fibres at this level (UNGERSTEDT, 1971; CONRAD & PFAFF, 1976; FONNUM *et al.*, 1974; LINDVALL & BJÖRKLUND, 1974).

Tissue preparation. Normal and operated animals were killed by decapitation, the lesioned animals 6–14 days after operation. The brain was rapidly removed and frozen on a microtome chuck with a CO_2 jet, and 40 μm serial sections from the telencephalic and mesencephalic regions under study were cut in a cryostate at about −16°C. The sections were freeze-dried and collected as previously described (FONNUM *et al.*, 1970). At regular intervals sections were collected on microscopic slides, allowed to dry and stained with methylene blue or for AChE as described by STORM-MATHISEN (1970). During the preparation of the sections, the hemitransection was examined under the microscope.

Dissection. Corresponding freeze-dried and stained sections were compared simultaneously under a stereo microscope. The different regions were identified from the atlas of KÖNIG & KLIPPEL (1963) and the parts to be analysed were dissected by the use of razor blade splints and weighed on a fish-pole quartz fibre balance (FONNUM *et al.*, 1970). In the telencephalon, we used sections from the level A 9400–A 8400 (KÖNIG & KLIPPEL, 1963) to dissect the olfactory tubercle, the nucleus accumbens, the septum and the striatum (Fig. 1). The olfactory tubercle was divided in two parts; a ventral part containing the olfactory tubercle proper and a dorsal part being contaminated by the islands of Calleja and perhaps by some fibres from the MFB.

The nucleus accumbens was divided into a medial and a lateral part by placing a vertical cut at the ventral tip of the lateral ventricle. The sample from septum was taken from the area adjacent to the medial boundary of the nucleus accumbens. Samples from striatum were taken out as indicated in Fig. 1.

Samples from the region traversed by MFB were initially dissected from sections between A 8400 and A 7900. Later this region was studied in sections from unoperated brains running from A 9500 to A 5900 (Fig. 2), and particular emphasis was placed on the sections at A 7200, where samples from lateral and medial preoptic area, nucleus tractus diagonalis and MFB could be distinguished (Fig. 3).

For subcellular fractionation and uptake studies samples from normal brains were used. Striatum, globus pallidus and MFB were dissected out from 300 μm sections prepared by a Sorvall tissue chopper at levels between A 7600 and A 6700. For MFB a sample corresponding to samples 3 and 4 in Fig. 3 was dissected.

In mesencephalon structures were dissected at level A 2000–A 1500 as indicated in Fig. 4. The ventral tegmental area was divided in three parts, the ventral, middle and dorsal. Substantia nigra was divided into pars compacta and pars reticulata.

Biochemical methods. DL-3,4-Dihydroxy[2-^{14}C]phenylalanine, L-[1-^{14}C]glutamic acid and [1-^{14}C]acetyl-CoA were obtained from the Radiochemical Centre, Amersham; [2,3-^{3}H(N)]γ-amino-butyric acid was from New England Nuclear, Boston.

ChAT was assayed according to FONNUM (1975). GAD was determined by the method of ALBERS & BRADY (1959)

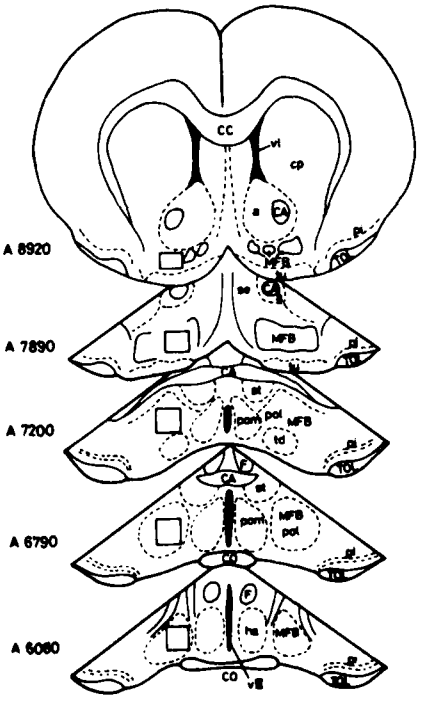

FIG. 2. Localization of samples taken out for analysis of medial forebrain bundle (MFB) in rostrocaudal direction. Drawings modified after KÖNIG & KLIPPEL (1963). Abbreviations: CC = corpus callosum; vl = lateral ventricle; cp = nucleus caudatus; a = nucleus accumbens; tu = tuberculum olfactorium; pi = cortex piriformis; TOL = tractus olfactorius lateralis; se = septum; CA = commissura anterior; st = nucleus interstitialis striae terminalis; pom = medial preoptic area; pol = lateral preoptic area; td = nucleus tractus diagonalis; F = fornix; CO = chiasma opticum; ha = nucleus anterior hypothalami; vIII = third ventricle.

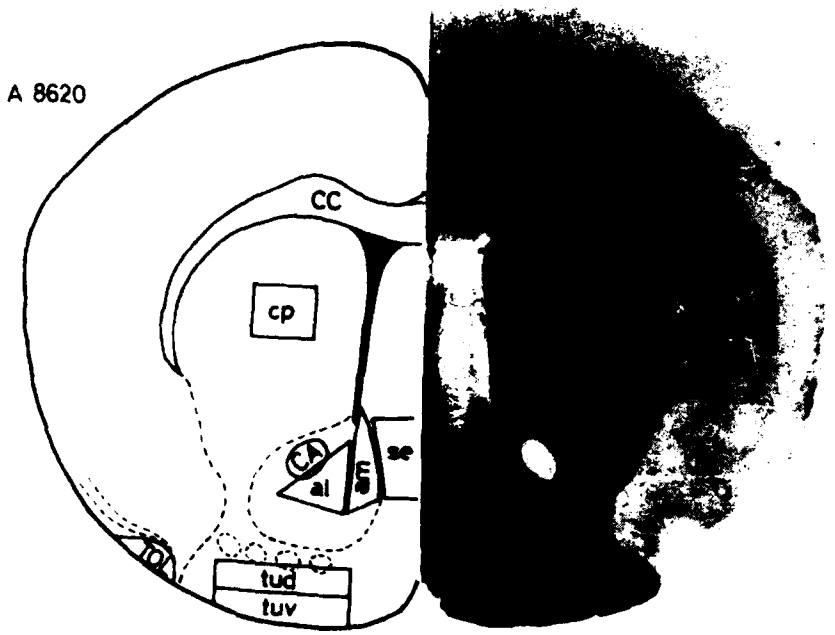

FIG. 1. Right side: Section from A 8620 (KÖNIG & KLIPPEL, 1963) stained for AChE. Left side: Localization of dissected samples. Abbreviations: cp = nucleus caudatus putamen; am = nucleus accumbens, medial part; al = nucleus accumbens, lateral part; se = septal sample; tuv = tuberculum olfactorium, ventral part; tud = tuberculum olfactorium, dorsal part; CA = commissura anterior; CC = corpus callosum; TOL = tractus olfactorius lateralis.

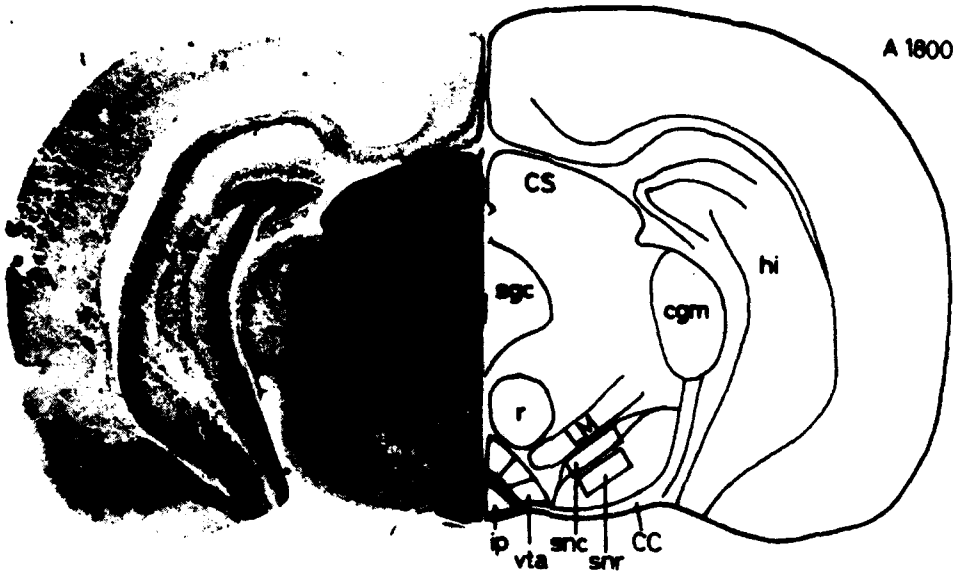

FIG. 4. Left side: Section from A 1800 (KÖNIG & KLIPPEL, 1963) stained for AChE. Right side: Localization of dissected samples. Abbreviations: CS = colliculus superior; cgm = corpus geniculatum mediale; sgc = substantia grisea centralis; r = nucleus ruber; LM = lemniscus medialis; hi = hippocampus; ip = nucleus interpeduncularis; vta = ventral tegmental area; snc = substantia nigra, pars compacta; snr = substantia nigra, pars reticulata; CC = crus cerebri.

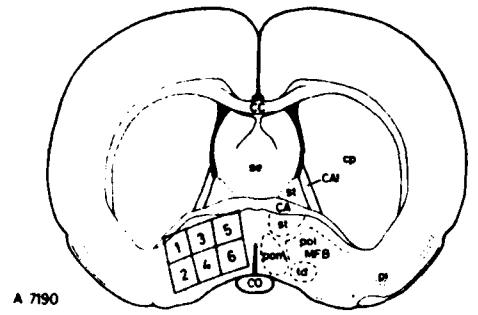

FIG. 3. Localization of samples dissected for study of medial forebrain bundle (MFB) in the frontal plane. Drawing modified after KÖNIG & KLIPPEL (1963). 1–6: Samples dissected. Abbreviations: CAI = capsula interna; else as in Fig. 2.

as described by HENKE & FONNUM (1976), except that the incubation temperature was 37 C. In samples dissected from fresh tissue (Table 5), the final glutamate concentration was reduced to 10 mM and the absolute activity found cannot be compared directly with those in Tables 3 and 4. AAD was assayed as described by BROCH & FONNUM (1972) using Kalignost extraction (FONNUM, 1969). The final concentration of L-DOPA was 0.3 mM. Protein was determined as described by LOWRY *et al.* (1951).

High affinity uptake was studied in normal brain. Samples were dissected from wet tissue and homogenized at 800 rev. min in a glass/Teflon homogenizer in 100 µl 0.32 M-sucrose (FONNUM *et al.*, 1970). 10 µl of the homogenate (5–30 µg protein) were added directly to 0.5 ml oxygenated incubation medium, which consisted of 15 mM-Tris–HCl buffer, pH 7.4, 140 mM-NaCl, 5 mM-KCl, 1.2 mM-$CaCl_2$, 1.2 mM-$MgSO_4$, 1.2 mM-Na_2–HPO_4, pH 7.4, and 10 mM-D-glucose. The tissue was preincubated for 3 min in a shaking incubator at 25°C, and the uptake started by addition of radioactive GABA (final concentration 1×10^{-7} M). The incubation was stopped after three minutes by rapid filtration through Millipore filters (0.45 µM pore size), followed by washing with 0.9% NaCl. The radioactivity was extracted in 10% Triton X-100 and after addition of scintillant, counted in a scintillation counter. Blanks were run similarly but using a sodium free incubation medium, where 280 mM-sucrose and 1.2 mM-Tris–phosphate buffer had replaced the sodium chloride and sodium phosphate.

The proportion of particulate GAD in normal brain was determined on samples dissected and homogenized as for 'high affinity uptake'. The homogenate was centrifuged at 17000 *g* for 1 h. GAD and protein was compared in supernatant and pellet.

RESULTS

Telencephalon

The AAD activity (Table 1) was highly concentrated in the striatum, the nucleus accumbens and the ventral part of the olfactory tubercle. In the medial part of nucleus accumbens, reported to contain more cells (POWELL & LEMAN, 1976) and staining more intensely for AChE (Fig. 1), we found a significantly higher level of activity than in the lateral part ($P < 0.001$). A high level of activity was also found in the region of the rostral MFB. After hemisection, the enzyme activity on the lesioned side decreased in all regions studied (Table 1), due to the degeneration of the rostral parts of the ascending aminergic fibres. In striatum, nucleus accumbens and olfactory tubercle, the AAD-activity of the lesion side was reduced more than 50%.

The ChAT activity (Table 2) was found to be very high in all regions examined, particularly in structures known to contain a high level of dopamine (striatum, nucleus accumbens and olfactory tubercle). The medial part of nucleus accumbens again contained a higher activity than the lateral part. Hemisection of the brain did not significantly reduce the activity in any of the structures studied (Table 2).

AChE-staining of the telencephalon (Fig. 1) resulted in a picture comparable to that described by JACOBOWITZ & PALKOVITS (1974). The highest AChE activity was located in the medial nucleus accumbens and the ventral olfactory tubercle, in good agreement with the high ChAT activity found in these structures. AChE-staining did not change after hemitransection.

The GAD activity (Table 3) was high in all limbic structures, particularly the medial part of nucleus accumbens, olfactory tubercle and septum. There was no loss of activity after hemitransection. The activity in the region of the rostral MFB, however, was very impressive, being comparable to the activity of the pars reticulata of the substantia nigra, which hitherto has been acknowledged to be the region with the highest GAD activity in mammalian brain (ALBERS & BRADY, 1959; FONNUM *et al.*, 1974; TAPPAZ *et al.*, 1976). A rostrocaudal survey of GAD in the MFB-region (Fig. 2) disclosed that this activity peaked in the sections between A 7900 and A 7000 (Fig. 5). Here the MFB emerging from the lateral hypothalamus, traverses the lateral preoptic area, and, passing the commissura anterior, enters the socalled substantia innominata. In the middle of this region, the GAD distribution in the frontal plane was further analysed (Fig. 3). The activity was found to be concentrated in a region immediately ventral to the commissura anterior, and to be clearly separated from the nucleus tractus diagonalis, the nucleus interstitialis striae terminalis and the medial preoptic area, all of which also contained high GAD activites (Table 4). Dissection of samples from fresh tissue confirmed that the GAD activity and GABA uptake were comparable to those in substantia nigra and higher than in globus pallidus (Table 5). Homogenisation and subsequent centrifugation of a sample from this region (parts 3 and 4, Fig. 3) demonstrated most of the enzyme to be localized in the particulate fraction (Table 5).

Mesencephalon

AAD was found to display a high activity in the middle and dorsal parts of the ventral tegmental area whereas a significantly lower activity was found in

TABLE 1. REGIONAL DISTRIBUTION OF AROMATIC AMINO ACID DECARBOXYLASE IN NORMAL AND TRANSECTED SIDE OF RAT BRAIN

Region	Aromatic amino acid decarboxylase: Unoperated (μmol/h/g dry wt)	Aromatic amino acid decarboxylase: Transected (Percent of normal brain)
Olfactory tubercle V	10.2 ± 2.2 (7,2)	37 ± 7 (13,8)*
Olfactory tubercle D	4.4 ± 1.0 (4,2)	50 ± 7 (2,2)*
N. Accumbens medial	16.6 ± 1.2 (10,3)	39 ± 5 (11,8)*
N. Accumbens lateral	10.4 ± 0.8 (14,3)	40 ± 6 (10,8)*
Septum	6.2 ± 0.6 (5,2)	33 ± 3 (2,1)*
Neostriatum	14.2 ± 1.0 (15,3)	14 ± 3 (11,8)*
Medial forebrain bundle	14.0 ± 2.6 (7,3)	18 ± 5 (2,2)*
N. Interpeduncularis	5.1 ± 0.8 (19,7)	102 ± 12 (21,7)
Ventral tegmental area D	24.4 ± 2.2 (10,3)	86 ± 15 (10,3)
Ventral tegmental area M	28.6 ± 4.6 (10,3)	95 ± 18 (8,3)
Ventral tegmental area V	13.2 ± 2.6 (6,3)	132 ± 14 (7,2)
Substantia nigra compacta	15.0 ± 2.6 (5,2)	99 ± 11 (7,2)
Substantia nigra reticulata	4.0 ± 0.6 (19,2)	74 ± 15 (11,7)

The results are expressed as mean ± S.E.M. (number of samples, number of animals). D = dorsal, M = middle, V = ventral.

* Operated side significant different from unoperated side, $P < 0.05$ (Student t-test).

Since we routinely measured the activity in n. accumbens, olfactory tubercle V and neostriatum to examine the extent of the lesion more data appear on the transected side than on the normal side.

the ventral part of this region ($P < 0.05$, t-test). Higher activity was found in the pars compacta than in pars reticulata of substantia nigra, and an intermediate activity was found in the interpeduncular nucleus (Table 1). After hemisection, no statistically significant changes occurred in any of these regions.

ChAT was highly concentrated in nucleus interpeduncularis. The activity was low in the ventral tegmental area and in substantia nigra (Table 2). The dorsal part of the ventral tegmental area contains fibres belonging to the cholinergic habenulo-interpeduncular tract (KATAOKA *et al.*, 1973), and this probably accounts for the fact that this region has a significantly ($P < 0.05$) higher activity than the ventral part. No significant changes occurred in these structures after hemisection (Table 2).

AChE-staining of the mesencephalon (Fig. 4) showed an intense staining of nucleus interpeduncularis in agreement with the high ChAT activity also present. The pars compacta of substantia nigra and

TABLE 2. REGIONAL DISTRIBUTION OF CHOLINE ACETYLTRANSFERASE IN NORMAL AND HEMITRANSECTED RAT BRAIN

Region	Choline acetyltransferase activity: Normal brain (μmol/h/g dry wt)	Operated brain: Unoperated side (Per cent of normal brain)	Operated brain: Transected side (Per cent of normal brain)
Olfactory tubercle V	96 ± 6 (14,4)	106 ± 9 (14,5)	114 ± 8 (10,3)
Olfactory tubercle D	71 ± 4 (7,4)	109 ± 13 (7,4)	104 ± 22 (6,3)
N. Accumbens medial	84 ± 17 (9,3)	106 ± 6 (21,7)	118 ± 14 (14,6)
N. Accumbens lateral	41 ± 8 (8,3)	120 ± 10 (21,7)	117 ± 10 (13,6)
Septum	45 ± 6 (11,3)	112 ± 14 (12,5)	96 ± 22 (8,3)
Neostriatum	83 ± 6 (7,3)	98 ± 5 (25,6)	102 ± 9 (17,5)
Medial forebrain bundle	52 ± 9 (9,4)	125 ± 17 (11,4)	117 ± 15 (10,4)
N. Interpeduncularis	311 ± 26 (5,3)	109 ± 9 (15,5)	122 ± 10 (15,5)
Ventral tegmental area D	18 ± 4 (8,3)	91 ± 13 (8,4)	83 ± 14 (9,4)
Ventral tegmental area M	10 ± 2 (7,3)	110 ± 20 (10,4)	112 ± 20 (10,4)
Ventral tegmental area V	7 ± 2 (7,3)	109 ± 28 (8,4)	103 ± 11 (9,4)
Substantia nigra reticulata + compacta	3 ± 1 (5,3)	110 ± 25 (10,2)	120 ± 28 (9,2)

The results are expressed as mean ± S.E.M. (number of samples, number of animals). Abbreviations as in Table 1.

TABLE 3. REGIONAL DISTRIBUTION OF GLUTAMATE DECARBOXYLASE IN NORMAL AND HEMITRANSECTED RAT BRAIN

Region	Glutamate decarboxylase activity		
	Normal brain (μmol/h/g dry wt)	Unoperated side (Per cent of normal brain)	Transected side (Per cent of normal brain)
Olfactory tubercle V	220 ± 21 (9,3)	103 ± 9 (12,5)	120 ± 11 (17,4)
Olfactory tubercle D	274 ± 5 (9,3)	108 ± 10 (10,5)	93 ± 8 (12,5)
N. Accumbens medial	220 ± 11 (11,3)	103 ± 15 (20,5)	110 ± 8 (19,5)
N. Accumbens lateral	163 ± 48 (9,3)	88 ± 21 (15,3)	92 ± 10 (13,5)
Septum	187 ± 6 (9,3)	117 ± 16 (12,4)	112 ± 5 (12,3)
Neostriatum	90 ± 11 (9,3)	114 ± 9 (18,5)	118 ± 12 (11,4)
Medial forebrain bundle	600 ± 88 (6,2)	110 ± 11 (5,3)	108 ± 17 (5,3)
N. Interpeduncularis	144 ± 10 (6,3)	99 ± 5 (18,7)	96 ± 5 (16,5)
Ventral tegmental area D	91 ± 8 (6,3)	97 ± 8 (19,5)	104 ± 11 (15,5)
Ventral tegmental area M	94 ± 8 (6,3)	96 ± 9 (16,5)	98 ± 12 (13,5)
Ventral tegmental area V	103 ± 9 (14,5)	94 ± 10 (14,5)	91 ± 8 (12,7)
Substantia nigra reticulata	615 ± 43 (6,3)	91 ± 6 (39,6)	19 ± 4 (30,6)*
Substantia nigra compacta	234 ± 41 (4,3)	109 ± 12 (19,3)	57 ± 5 (13,3)*

The results are expressed as mean ± S.E.M. (number of samples, number of animals). Abbreviations as in Table 1.
* Significantly different from normal brain $P < 0.01$ (Student t-test).

the ventral tegmental area displayed intermediate staining, which is in contrast to the low ChAT activity in these regions and further demonstrates that the AChE is unreliable as a cholinergic marker in this region, in agreement with other reports (FONNUM, 1973*a*, FONNUM *et al.*, 1974).

Higher activity of GAD was found in pars reticulata than in pars compacta of substantia nigra. Substantia nigra contained much higher activity than any part of the ventral tegmental area and nucleus interpeduncularis. Hemisection was accompanied by a dramatic loss of GAD in both parts of substantia

TABLE 4. DISTRIBUTION OF GAD ACTIVITY IN THE REGION TRAVERSED BY THE MEDIAL FOREBRAIN BUNDLE

Sample number according to Fig. 3	GAD activity (μmol/h/g dry wt)
1	612 ± 32
2	715 ± 108
3	1042 ± 76*
4	757 ± 96
5	502 ± 46
6	508 ± 98

The results are expressed as mean ± S.E.M. and are taken from 6–8 samples.
* Sample 3 significantly higher than the other samples ($P < 0.05$).

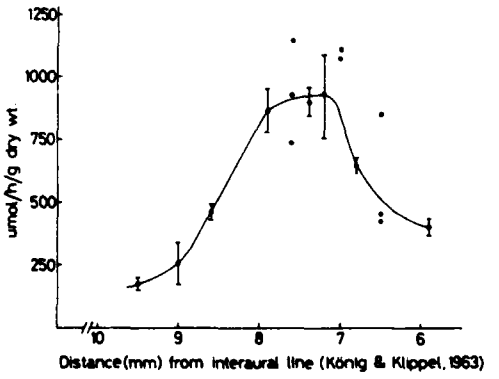

FIG. 5. Distribution of GAD activity in the MFB in rostrocaudal direction. Each point is the mean ± S.E.M. of 4–14 analyses. If less samples were analyzed, individual values are given.

TABLE 5. TOTAL GAD ACTIVITY, PARTICULATE GAD ACTIVITY AND HIGH AFFINITY GABA UPTAKE OF HOMOGENATE FROM SOME REGIONS RICH IN GABA

	Total GAD activity (μmol/h/g protein)	Particulate GAD activity (Per cent of total)	GABA uptake (pmol/min/mg protein)
Medial forebrain bundle	715 ± 110	79 ± 4	15.2 ± 1.2
Globus pallidus	361 ± 28	79 ± 1	6.3 ± 1.2
Striatum	161 ± 32	79 ± 2	4.3 ± 0.5
Substantia nigra	581 ± 82	85 ± 5	12.1 ± 0.8

The results are mean ± S.E.M. from 3 experiments.

nigra on the lesioned side, but it was without effect on GAD activities in the ventral tegmental area or in nucleus interpeduncularis (Table 3).

DISCUSSION

In the present report we have studied the levels of transmitter synthesising enzymes as markers for cholinergic, GABAergic and aminergic structures. The advantage of studying ChAT and GAD instead of ACh and GABA has been discussed elsewhere (FONNUM, 1973*b*). AAD is, however, involved in the synthesis of both dopamine, noradrenaline and serotonin (KUNTZMAN *et al.*, 1961). Immunofluorescence shows, however, particular intense staining of AAD in dopaminergic terminals, rather than in serotonergic or noradrenergic neurons (HÖKFELT *et al.*, 1975). Since the present study involves regions where the level of dopamine predominates over noradrenaline and serotonin (BROWNSTEIN *et al.*, 1974; HORN *et al.*, 1974; SAAVEDRA *et al.*, 1974), the level of AAD thus probably mainly reflects dopaminergic structures. It is well established that the dopaminergic fibres are derived from the socalled A9 and A10 cell groups in the mesencephalon (DAHLSTRÖM & FUXE, 1964; UNGERSTEDT, 1971), and the decrease of AAD in the telencephalon was therefore taken as evidence for a successful lesion. In all operated animals, AAD was decreased by more than 50% on the lesion side.

Previous studies have revealed that the *nucleus accumbens* contains a high concentration of dopamine (BROWNSTEIN *et al.*, 1974; HORN *et al.*, 1974), tyrosine hydroxylase (SAAVEDRA & ZIVIN, 1976), GAD (TAPPAZ *et al.*, 1976) and ChAT (PALKOVITS *et al.*, 1974). These findings have been substantiated and expanded. The highest concentration of AAD, GAD and ChAT was localized to the medial part, which is particularly cell rich, of the nucleus (POWELL & LEMAN, 1976). This part also stained more intensely for AChE and with catecholamine fluorescence (JACOBOWITZ & PALKOVITS, 1974). Neither ChAT nor GAD changed significantly after hemisection, excluding the possibility that these elements derived from ascending fibres.

Also the *olfactory tubercle* contains a high level of dopamine (BROWNSTEIN *et al.*, 1974; HORN *et al.*, 1974) and a dense distribution of dopaminergic terminals (UNGERSTEDT, 1971). GAD and ChAT have also been found in considerable concentrations (KATAOKA *et al.*, 1975; PALKOVITS *et al.*, 1974; TAPPAZ *et al.*, 1976). Whereas the highest level of AAD was confined to the ventral part, only moderate differences were found for ChAT and for GAD slightly more was found in the dorsal part. The latter may be due to slight contamination of the dorsal part with the MFB. Previous work has suggested that the cholinergic input to the olfactory tubercle may be derived from cells in the lateral preoptic area (LEWIS & SHUTE, 1967). If a substantial part of the cholinergic input had been derived from this area, we would have expected to find a substantial decrease of ChAT in the olfactory tubercle since our lesion destroyed at least the caudal part of the preoptic area.

The distribution of AAD, GAD and ChAT have been previously studied in detail in *neostriatum* (caudatus–putamen) (FONNUM *et al.* in press (*b*)). The level of AAD and ChAT was similar to the levels in the mesolimbic areas, whereas GAD was significantly lower in the dorsal striatal sample than in the mesolimbic areas. ChAT and GAD did not decrease after hemitransection. The findings in striatum after hemitransection are in good agreement with the suggested ascending dopaminergic fibres (ANDÉN *et al.*, 1964), cholinergic interneurons (GUYENET *et al.*, 1975; MCGEER *et al.*, 1974) and descending GABAergic fibres (KIM *et al.*, 1971; FONNUM *et al.*, 1974, 1977*b*).

The localization of ChAT and GAD in different parts of *septum* have been studied previously (PALKOVITS *et al.*, 1974; TAPPAZ *et al.*, 1976). We included septum in our study since it also receives dopaminergic fibres from the A10 group (LINDVALL, 1975). The sample which included the nucleus of the diagonal band and the medial septum contained less AAD and ChAT activities than the other regions examined, but relatively high GAD activity. Interestingly, ChAT and GAD, unlike AAD, did not decrease after hemisection.

Several interesting results were found in the samples from the rostral part of the *medial forebrain bundle*. This bundle consists in part of serotonergic, dopaminergic and noradrenergic fibres (MOORE *et al.*, 1965; UNGERSTEDT, 1971), and we found a high level of AAD in the region, in keeping with the role of the enzyme in the synthesis of both catecholamines and serotonin. After hemisection, the activity decreased as expected. The level of ChAT was intermediate, in agreement with the diffuse AChE staining. Since ChAT was unchanged after transection, there are probably no cholinergic fibres in the bundle. The most interesting result in this region, however, was the unexpectedly high level of GAD, which in the freeze-dried sections was well above the activity of GAD in the substantia nigra. In the fresh tissue sections (Table 5), the dissection encompassed a larger region, but the activity was still well above the level both in substantia nigra and in globus pallidus, regions with high GAD activity (FONNUM *et al.*, in press (*b*)). Several research groups have found high enzyme activities in neighbouring regions such as the lateral and medial preoptic areas (TAPPAZ *et al.*, 1976; KATAOKA *et al.*, 1975), or the bed nucleus of the stria terminalis (BEN-ARI *et al.*, 1976). These results have been confirmed, but they do not account for the high activity in the MFB. Our results demonstrate that this maximum is located in a region immediately ventral to the commissura anterior and traversed by the MFB. The most caudal part corresponds to the rostral part of the lateral preoptic area. This is in agreement with the distribution of GABA in the lateral hypothalamus, which was found to be highly concentrated at the A 7000 level (KIMURA & KURIYAMA,

1975). The region of high GAD activity is not limited to a single nucleus delineated by classical anatomical methods. The closest may be the substantia innominata which, however, is ill-defined in rodents. The latter region has been suggested to represent the ventral part of globus pallidus (HEIMER & WILSON, 1975). Our findings on the rostral part of MFB seem to be in agreement with such a suggestion since both MFB and globus pallidus contain high GAD activities, active high affinity uptake of GABA and moderate ChAT activities (Table 5; FONNUM *et al.*, in press (*b*)). Examination of GAD activity in more rostral and caudal parts of the MFB demonstrated a distinct decline in activity, in agreement with TAPPAZ *et al.* (1976), who only found an intermediate level of GAD in the MFB dissected from hypothalamus. Thus the high activity found was probably not due to MFB fibres. In agreement with this, no change of activity was found after hemitransection, indicating that the MFB does not contain GABAergic ascending fibres.

In the mesencephalon, the ventral tegmental area contains the dopaminergic cell bodies which give rise to the 'mesolimbic' fibres (UNGERSTEDT, 1971; BJÖRKLUND & LINDVALL, in press; SIMON *et al.*, 1976). Since the functional subgroups of these cells are differently topographically organized, we divided the area into three, i.e. the dorsal part which projects mainly to septum, the middle part which mainly projects to nucleus accumbens, and the ventral part which mainly projects to olfactory tuberculum (BJÖRKLUND & LINDVALL, *in press*). The three parts of the ventral tegmental region did not differ markedly, particularly when one remembers that the ventral sample also included the area near the brain surface which contains few dopaminergic cells (HÖKFELT *et al.*, 1975). In the substantia nigra, as expected, we found the dopaminergic cells to be concentrated in the pars compacta. The ChAT levels in both these dopaminergic cell nuclei were low. The GAD activity, however, was differently distributed. The activity in the substantia nigra was 5 to 10 times higher than in the ventral tegmental area. Furthermore, whereas the GAD level on the lesioned side was reduced in the substantia nigra after hemitransection, the activity in the ventral tegmental area was unchanged. We therefore conclude that the mesolimbic dopaminergic cells do not receive a descending GABAergic innervation, as do the dopaminergic cells in the substantia nigra.

In conclusion there were many similarities between the distribution of GAD, ChAT and AAD in the limbic and neostriatal structures examined, but there were considerable differences in both the origin as well as the level of GABAergic fibres in the two regions containing dopaminergic cell bodies.

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PAPER II

THE EFFECTS OF SURGICAL AND CHEMICAL LESIONS ON NEUROTRANSMITTER CANDIDATES IN THE NUCLEUS ACCUMBENS OF THE RAT

I. WALAAS and F. FONNUM

Norwegian Defence Research Establishment, Division for Toxicology, Box 25, N-2007 Kjeller, Norway

Abstract The origin of fibers containing different neurotransmitter candidates in the nucleus accumbens of rat brain has been studied with surgical and chemical lesion techniques. Destruction of the medial forebrain bundle decreased the activity of aromatic amino acid decarboxylase by 80%, in the nucleus. Cutting of the fornix or a hemitransection decreased the high affinity uptake of glutamate by 45%, and the endogenous level of glutamate by 33%. The high affinity uptake of glutamate was concentrated in the synaptosomal fraction and the decrease after the lesion was most pronounced in this fraction. Restricted lesions indicated that fibers in the fimbria fornix coming from the subiculum were responsible for this part of the glutamate uptake in the nucleus. Local injection of kainic acid into the nucleus was accompanied by a 75% decrease in choline acetyltransferase and a 35% decrease in acetylcholinesterase activities, a 70% decrease in glutamate decarboxylase activity and a 60% decrease in the high affinity uptake of γ-aminobutyrate, a 45% decrease in high affinity glutamate uptake, and no change in aromatic amino acid decarboxylase activity. Performing a lesion of the fornix after kainic acid injection led to an 85% decrease in high affinity glutamate uptake, without further affecting the other neuronal markers.

The results indicate that all aminergic fibers to the nucleus accumbens are ascending in the medial forebrain bundle, that the subiculum–accumbens fibers are 'glutamergic', and that the nucleus also contains intrinsic glutamergic or aspartergic cells. Cholinergic and γ-aminobutyrate-containing cells are wholly intrinsic to the nucleus.

THE NUCLEUS accumbens septi has in recent years been the subject of a number of morphological and developmental studies which have indicated that the nucleus and its connections have characteristics in common both with 'limbic' structures and with the basal ganglia (HEIMER & WILSON, 1975; SWANSON & COWAN, 1975; CONRAD & PFAFF, 1976; POWELL & LEMAN, 1976; WILLIAMS, CROSSMAN & SLATER, 1977; LAWSON, MAY & WILLIAMS, 1977). Also, the electrophysiological and neurochemical interactions between several transmitter candidates present in the nucleus, e.g. dopamine, acetylcholine, glutamate and γ-aminobutyric acid (GABA), have attracted attention (WOODRUFF, MCCARTHY & WALKER, 1976; MCCARTHY, WALKER & WOODRUFF, 1977; BARTHOLINI, 1977; CONSOLO, LADINSKY, BIANCHI & GHEZZI, 1977; MAO, CHENEY, MARCO, REVUELTA & COSTA, 1977; PÉREZ DE LA MORA & FUXE, 1977). However, except for the ascending dopaminergic fibers (LINDVALL & BJÖRKLUND, 1974) our knowledge of the anatomical organization of the fibers containing these transmitters is still incomplete.

We have previously shown that in the rat, the nucleus contains high densities of putative aminergic [mostly dopaminergic and serotonergic (HORN, CUELLO & MILLER, 1974; SAAVEDRA, BROWNSTEIN & PALKOVITS, 1974)], cholinergic and GABAergic structures, especially in the medial part (FONNUM, WALAAS & IVERSEN, 1977). It was shown that a hemitransection caudal to the nucleus destroyed the aminergic fibers which travel in the medial forebrain bundle (LINDVALL & BJÖRKLUND, 1974; PALKOVITS, 1977), but left the cholinergic and GABAergic structures intact (FONNUM *et al.*, 1977). In the present study, the precise origin of these cholinergic, GABAergic and also possible 'glutamergic' fibers has been investigated with surgical and chemical lesions.

A preliminary account of some of these results has been given (WALAAS & FONNUM, 1977).

Abbreviations: AChE, acetylcholinesterase; ChAT, choline acetyltransferase; GABA, γ-aminobutyrate.

EXPERIMENTAL PROCEDURES

Materials

Kainic acid was from Sigma Chemical Co., St Louis, U.S.A. White Wistar rats of both sexes, weighing 170–200 g, were from Dyrlaege Mollergaard-Hanssens Avlslaboratorium, Denmark. Radiochemicals were obtained from New England Nuclear, Boston, U.S.A. and The Radiochemical Centre, Amersham, U.K.

Lesions

The animals were anesthetized with a mixture of diazepam and Hypnorm veterinary (Janssen Pharmacological Co.) and placed in a Kopf stereotaxic apparatus. The skull was opened with a dental drill, and known afferents to the nucleus accumbens (DE OLMOS & INGRAM, 1972; ITO, ISHIDA, MIYAKAWA & NAITO, 1974; POWELL & LEMAN, 1976; SWANSON & COWAN, 1977) were lesioned.

(1) A *hemitransection* was done with a blunt spatula at the level of the bregma, as previously described (FONNUM

et al., 1977). This lesion destroyed all ipsilateral ascending fibers to the nucleus.

(2) The *medial forebrain bundle* was cut by lowering a thin spatula at the level of the bregma to the base of the brain 1.5–3 mm lateral to the midline. This lesion cut through globus pallidus and the median forebrain bundle fibers, but left the fornix bundle intact.

(3) Fibers from the *thalamus* were destroyed by placing an electrode insulated to the tip at coordinates A: 4.0, L: 1.0, V: −0.2 (KONIG & KLIPPEL, 1963), and making an electrocoagulation with 2 mA for 20 s. This led to a lesion centered in the parafascicular and centromedian nuclei.

(4) The *dorsal neocortex* was removed on one side by suction down to the white matter under visual guidance, reaching within 2–3 mm from the frontal pole and including the whole cingular cortex.

(5) The *caudal pyriform cortex* was exposed after removal of the zygomatic arch and ventral skull (POWELL, COWAN & RAISMAN, 1965) and lesioned by suction under visual guidance. This lesion destroyed the cortex between the rhinal fissure and the base of the brain, but left the amygdaloid nuclei intact.

(6) '*Fornix lesion*', which comprised the cingulum bundle, fornix and fornix superior and stria terminalis, was done with a scalpel 2 mm behind the bregma, lowered 6 mm from the skull surface and moved 4 mm from the midline on both sides (Fig. 1). To differentiate between the fiber bundles destroyed by this lesion, a series of more restricted lesions were performed:

(7) The *cingulum bundle* was cut bilaterally with a scalpel just in front of the bregma, lowering it 5 mm from the skull surface (Fig. 1).

(8) A *long parasagittal lesion* which comprised the stria terminalis, the lateral alveus-fimbria system and also the ventral amygdalofugal pathway was done with a blunt cannula lowered to the base of the brain 3 mm lateral to the midline, moved in the sagittal plane from A: 1.0 to A: 6.0, and then laterally to the side of the brain (Fig. 1).

(9) A *short parasagittal lesion* which comprised the initial part of the stria terminalis, the ventral amygdalofugal fibers and also a small part of the anteroventral part of the fimbria, was done with a cannula in the same way, but moving it rostrally only from A: 3.0 to A: 6.0 (Fig. 1).

(10) A *dorsal parasagittal lesion* which comprised the dorsal stria terminalis and lateral fimbria was done with a scalpel 2.5 mm lateral to the midline, lowered 6 mm from the skull surface and moved from the level of the bregma to the level of the lambda, terminating 4 mm from the skull surface. This lesion left the *medial and caudal part* of the hippocampal formation intact.

Kainic acid was dissolved in 0.9% NaCl (4 mg ml^{-1}) and the pH adjusted to 7.2 with NaOH. A hole was made in the skull, a Hamilton microsyringe was lowered to coordinates A: 9.0, L: 1.0, D: −0.9 (KONIG & KLIPPEL, 1963) and 0.5 µl was infused during a period of 1.5–2 min. Sham injections were performed by infusing 0.9% NaCl in the same manner. The kainic acid lesion was controlled on sections from separate brains stained for cells with May-Grünwald Giemsa stain.

To study the effect of combined chemical and surgical lesions, animals were injected with kainic acid, allowed to recover for 1 week and then operated with a bilateral fornix lesion.

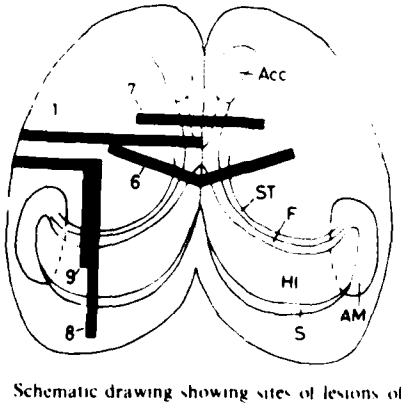

FIG. 1. Schematic drawing showing sites of lesions of different projections to the nucleus accumbens as viewed from above. 1, hemitransection; 6, fornix lesion; 7, cingulum bundle lesion; 8, long parasagittal lesion; 9, short parasagittal lesion. *Abbreviations*: ACC, nucleus accumbens; AM, amygdala; HI, hippocampus; S, subiculum; ST, stria terminalis; F, fimbria.

Tissue preparation

For *enzyme activity or uptake studies*, normal or lesioned animals were decapitated, the brain taken out and cut in 400–600 µm thick slices with a Sorvall tissue chopper in the cold room. All lesions were inspected, and the brains discarded if necessary. The nucleus accumbens, and, in normal brains, the olfactory tubercle, lateral septum, caudate putamen (neostriatum) and globus pallidus were dissected following the atlas of KONIG & KLIPPEL (1963). The samples were homogenized in glass–Teflon homogenizers at 800 rev. min in cold 0.32 M sucrose (2% w/v) so as to preserve synaptosomes (FONNUM, 1970), kept on ice and analyzed for uptake activity within 30 min. For *amino acid analysis*, the animals were killed by microwave irradiation focussed on the skull for 3 s (GUDELL CHESY, TRABUCCHI, DOTTI CHI, WANG & HAWKINS, 1974) and the nucleus accumbens dissected out. For *subcellular fractionation*, one nucleus accumbens (approx. 10 mg wet weight) was homogenized in 0.5 ml isotonic sucrose and separated into a P_2-pellet and mitochondrial, synaptosomal, myelin and soluble fractions as previously described (LUND KARLSEN & FONNUM, 1978). The synaptosomal marker choline acetyltransferase (ChAT, EC 2.3.1.6) the mitochondrial marker carnitine acetyltransferase (EC 2.3.1.7), the glutamate uptake and protein content were measured in P_2 and all fractions.

Biochemical analysis

For *uptake studies*, 5 µl of homogenate were added to 0.5 ml Tris–Krebs medium, incubated with radioactive ligand for 3 min at 25°C, and the uptake terminated by Millipore filtration as previously described (FONNUM *et al.*, 1977). As ligand, either L-[2,3-^{3}H]glutamate (10^{-7} M) or a mixture of [^{3}H]glutamate (10^{-7} M) and [U-^{14}C]GABA (10^{-7} M) were used. Under these conditions, glutamate and GABA do not interfere with each other's transport (BALAR & JOHNSTON, 1972). Separate experiments demonstrated that the radioactivity accumulated in nucleus accumbens homogenates was proportional to the amount of tissue within the range used in this study. In a few experiments, D-[2,3-^{3}H]aspartate was used as a non-metabolic ligand (DAVIES & JOHNSTON, 1976) to exclude the possibility of metabolic influence on the uptake.

TABLE 1. THE EFFECT OF HEMITRANSECTION ON NEUROTRANSMITTER MARKERS IN THE NUCLEUS ACCUMBENS

	Unoperated side (μmol h g protein)	Operated side (μmol h g protein)	Difference (%)
Glutamate uptake	2.61 ± 0.13 (14)	1.42 ± 0.16 (14)	−45*
GABA uptake	5.48 ± 0.36 (7)	4.87 ± 0.56 (7)	−11
Aromatic amino acid decarboxylase	35.6 ± 6.5 (9)	7.1 ± 2.3 (9)	−80*
Choline acetyltransferase	116 ± 10 (8)	100 ± 20 (8)	−15
Glutamate decarboxylase	310 ± 31 (6)	296 ± 51 (6)	−5

Results presented as mean ± S.E.M. (number of animals).
* $P < 0.001$ (Wilcoxon paired comparison test).

For *enzyme analysis*, the homogenates were treated with Triton X-100 (final conc. 0.2%, v/v). Previously described methods were used for determinations of ChAT (FONNUM, 1975*a*), aromatic amino acid decarboxylase (EC 4.1.1.26) (BROCH & FONNUM, 1972), glutamate decarboxylase (EC 4.1.1.15) (FONNUM *et al.*, 1977) and acetylcholinesterase (AChE, EC 3.1.1.7) (FONNUM, 1969). Carnitine acetyltransferase activity was analyzed using Kalignost extraction at acid pH to isolate the product (LUND KARLSEN & FONNUM, 1978).

Endogenous amino acids were analyzed by the double isotope dansylation technique as previously described (FONNUM & WALAAS, 1978). Protein was measured according to LOWRY, ROSEBROUGH, FARR & RANDALL (1951).

Statistical treatment

Comparisons between the sides with and without lesions or brains with and without lesions were performed with the Wilcoxon paired comparison test or the Wilcoxon two sample test as appropriate (HODGES & LEHMANN, 1964).

RESULTS

To study afferent transmitters in the nucleus accumbens, surgical lesion techniques were employed. Seven days after a hemitransection through the globus pallidus, the high affinity glutamate uptake was decreased by 45%, and the aromatic amino acid decarboxylase activity was decreased by 80%, while glutamate decarboxylase and ChAT activities and the high affinity uptake of GABA were unchanged (Table 1). Samples from the unoperated side displayed the same glutamate and GABA uptakes as sham operated and unoperated animals (data not shown). This has also been shown to be the case for aromatic amino acid decarboxylase, ChAT and glutamate decarboxylase (FONNUM *et al.*, 1977), and the unlesioned side was therefore used as control in the rest of this study, except where bilateral lesions were performed.

Further analysis of the glutamate uptake demonstrated that the decrease following hemitransection was almost maximally developed after 7 days (Fig. 2). Subcellular fractionation demonstrated that the uptake was concentrated in the synaptosomal fraction, where most of the decrease after lesion was also found (Fig. 3). In contrast, the mitochondrial marker carni-

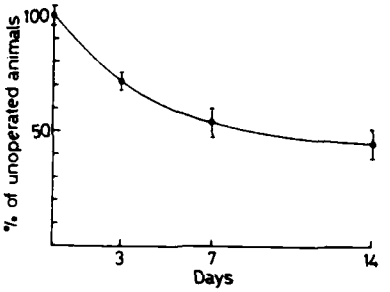

FIG. 2. Time course of changes in high affinity glutamate uptake in the nucleus accumbens after hemitransection. Each point is mean ± S.E.M. of five to nine operated animals expressed as per cent of simultaneously analyzed unoperated animals.

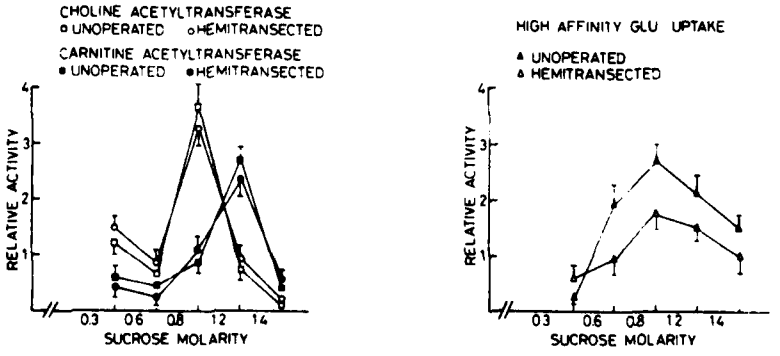

FIG. 3. Sucrose density gradient centrifugation showing the subcellular distribution of choline acetyltransferase, carnitine acetyltransferase and high affinity glutamate uptake in the nucleus accumbens of unoperated and hemitransected rat brain. Each point is mean ± S.E.M. from three animals expressed as relative to the activity in P_1-pellet from unoperated animals (see Experimental Procedures).

tine acetyltransferase (McCAMAN, McCAMAN & STAFFORD, 1966) and the synaptosomal marker ChAT did not display decreased activities in any of the fractions studied. Topographical distribution studies demonstrated that glutamate was most active in the neostriatum, slightly less so in the lateral septum and nucleus accumbens, rather low in the olfactory tubercle and very low in the globus pallidus (Table 2).

Selective lesions of known afferents to the nucleus accumbens (Fig. 1) revealed that lesions of the medial forebrain bundle, the thalamic nuclei, the dorsal neocortex, the caudal pyriform cortex and the cingulum bundle had no effect on glutamate uptake in the nucleus (Table 3). A short parasagittal lesion, which destroyed the stria terminalis and the ventral amygdalofugal pathway, or a dorsal parasagittal lesion, which destroyed the stria terminalis and the lateral fimbria, but left the ventrocaudal part of the hippocampal formation intact, also did not decrease the glutamate uptake. A long parasagittal lesion, however, which cut through the whole hippocampal region, led to a significant decrease in glutamate uptake. A fornix lesion, designed to destroy all fibers coming from the hippocampal formation, led to a decrease comparable to that found after hemitransection. These results indicate that part of the glutamate uptake in the nucleus accumbens is dependent on fibers which originate in the hippocampal formation and travel through the fornix. Aromatic amino acid decarboxylase activity analysis after some of these lesions confirmed that the decrease in glutamate uptake was independent of the aminergic innnervation, as a median forebrain bundle lesion decreased the aromatic amino acid decarboxylase activity by the same amount as a hemitransection without affecting the glutamate uptake (Table 3).

Amino acid analysis after a lesion of the fornix demonstrated a significant decrease in the concentration of endogenous glutamate, and also a slight decrease in aspartate, which, however, was barely significant ($P = 0.05$ with the Wilcoxon two-sample test, $P > 0.10$ with the Student's t-test). The levels of GABA, glycine and glutamine were essentially unchanged (Table 4).

To study the intrinsic transmitters in the nucleus accumbens, the neurotoxic glutamate analogue kainic acid was injected stereotactically into the nucleus. Histological staining of sections from these animals 5–6 days after injection (not shown) demonstrated a heavy loss of cells with large nuclei and basophil cytoplasm while the remaining cells were characterized by small dark nuclei, thus probably being of a glial nature. This lesion was not completely confined to the nucleus accumbens, but in most animals also encroached on adjoining regions of the septum, neostriatum and olfactory tubercle. Biochemical analysis after this lesion demonstrated approximately a 70% decrease in the activities of ChAT and glutamate decarboxylase, a 60% decrease in GABA uptake and a 35% decrease in AChE activity, while the aromatic amino acid decarboxylase activity was unchanged. The glutamate uptake was found to be decreased by approximately 45% (Table 5). In sham injected animals, no decrease in these parameters was found.

Performing both kainic acid injection and fornix transection in the same animals led to approximately an 85% decrease in the glutamate uptake. This treatment had no additional effect on ChAT, glutamate decarboxylase or aromatic amino acid decarboxylase activities compared to kainic acid alone (Table 6). Thus, the decreases in glutamate uptake after these two lesions were found to be additive.

TABLE 2. THE DISTRIBUTION OF THE UPTAKE OF 10^{-4} M GLUTAMATE IN THE BASAL GANGLIA AND 'MESOLIMBIC' REGIONS

	Glutamate uptake (μmol h g protein)
Neostriatum	4.14 ± 0.18 (27)
Globus pallidus	0.46 ± 0.07 (5)
Lateral septum	2.62 ± 0.27 (13)
Nucleus accumbens	2.67 ± 0.16 (15)
Olfactory tubercle	1.42 ± 0.12 (12)

Results presented as mean ± S.E.M. (number of animals).

TABLE 3. THE EFFECT OF RESTRICTED LESIONS ON THE UPTAKE OF GLUTAMATE AND ON THE ACTIVITY OF AROMATIC AMINO ACID DECARBOXYLASE IN THE NUCLEUS ACCUMBENS

Region with lesion	Glutamate uptake (per cent of control brain)	Aromatic amino acid decarboxylase (per cent of control brain)
Dorsal cortex	99 ± 7 (7)	
Thalamic nuclei	105 ± 13 (8)	
Medial forebrain bundle	86 ± 15 (8)	21 ± 7 (8)*
Pyriform cortex	111 ± 13 (4)	
Bilateral fornix	48 ± 4 (11)*	
Cingulum bundle	93 ± 11 (5)	
Dorsal parasagittal lesion	97 ± 4 (4)	91 ± 6 (4)
Short parasagittal lesion	88 ± 13 (4)	87 ± 8 (4)
Long parasagittal lesion	67 ± 16 (5)**	97 ± 9 (5)

Results presented as mean ± S.E.M. (number of animals).
* $P < 0.001$, ** $P < 0.05$ (Wilcoxon two-sample test).

TABLE 4. THE EFFECT OF FORNIX TRANSECTION ON THE CONCENTRATIONS OF SOME AMINO ACIDS IN THE NUCLEUS ACCUMBENS

	Unoperated side $n = 6$ (μmol g protein)	Lesioned side $n = 8$ (μmol g protein)	Δ%
Glutamate	103.4 ± 7.7	69.4 ± 6.3	−33*
Aspartate	25.6 ± 2.1	20.0 ± 2.1	−22**
Glycine	14.1 ± 1.9	15.5 ± 4.4	+10
γ-aminobutyrate	44.6 ± 3.6	39.0 ± 3.5	−13
Glutamine	94.5 ± 5.8	86.0 ± 9.9	−9

Results presented as mean ± S.E.M.
* $P < 0.02$, ** $P = 0.05$ (Wilcoxon two-sample test), or $0.10 < P < 0.20$ (Student's t-test).

TABLE 5. THE EFFECT OF LOCAL KAINIC ACID INJECTION ON NEUROTRANSMITTER MARKERS IN THE NUCLEUS ACCUMBENS

	Uninjected side	Injected side	Difference
	(μmol/h g protein)		(%)
Glutamate uptake	2.49 ± 0.25 (9)	1.32 ± 0.21 (9)	−47**
GABA uptake	4.00 ± 0.41 (5)	1.52 ± 0.25 (5)	−62*
Aromatic amino acid decarboxylase	39.4 ± 1.8 (9)	41.6 ± 2.1 (9)	+6
Acetylcholinesterase	7340 ± 440 (6)	4760 ± 800 (6)	−35*
Choline acetyltransferase	129 ± 7 (13)	31 ± 5 (13)	−76***
Glutamate decarboxylase	245 ± 20 (10)	75 ± 24 (10)	−70***

Results presented as mean ± S.E.M. (number of animals).
* $P = 0.03$, ** $P < 0.002$, *** $P < 0.001$ (Wilcoxon paired comparison test).

TABLE 6. THE EFFECT OF UNILATERAL KAINIC ACID INJECTION FOLLOWED BY BILATERAL LESION OF THE FORNIX ON NEUROTRANSMITTER MARKERS IN THE NUCLEUS ACCUMBENS

	Unlesioned animals (μmol h g protein)	Uninjected side (μmol h g protein)	Change (%)	Injected side (μmol h g protein)	Change (%)
Glutamate uptake	3.17 ± 0.20	1.87 ± 0.22	−41**	0.54 ± 0.16	−83*
Aromatic amino acid decarboxylase	40.7 ± 2.4	42.8 ± 2.4	+5	41.7 ± 3.2	+3
Choline acetyltransferase	102 ± 9	96 ± 7	−6	22 ± 7	−78*
Glutamate decarboxylase	256 ± 30	259 ± 30	+1	73 ± 27	−72*

Results presented as mean ± S.E.M. from six unlesioned and six operated animals.
* $P < 0.001$, ** $P = 0.02$ (compared with unlesioned animals, Wilcoxon two-sample test).

DISCUSSION

Methodological considerations

The suitability of aromatic amino acid decarboxylase, ChAT and glutamate decarboxylase as markers for catecholaminergic indolaminergic, cholinergic and GABAergic fibers respectively has previously been discussed (FONNUM, 1975*b*; FONNUM *et al.*, 1977). In contrast, a marker for putative glutamergic fibers has been difficult to find. However, recent reports indicate that the so-called 'high affinity uptake' of glutamate is specifically located in such terminals (YOUNG, OSTER-GRANITE, HERNDON & SNYDER, 1974; STORM-MATHISEN, 1977; DIVAC, FONNUM & STORM-MATHISEN, 1977; MCGEER, MCGEER, SCHERER & SINGH, 1977; LUND KARLSEN & FONNUM, 1978; FONNUM & WALAAS, 1978). This uptake might therefore be a suitable marker for such neurons, but at least *in vitro* the carrier does not seem to be able to distinguish between glutamate and aspartate, another transmitter candidate (BALCAR & JOHNSTON, 1972). In this study, we have therefore used glutamate uptake as marker for both putative glutamergic and aspartergic fibers, and performed amino acid analyses to differentiate between them.

Transmitters in afferent fibers

Judging from neuroanatomical studies, most fibers reaching the nucleus accumbens in rat brain seem to originate from more caudal regions (SWANSON & COWAN, 1975). We therefore started these studies with a complete hemitransection placed caudal to the nucleus. This has previously been shown to be without effect on glutamate decarboxylase and ChAT activities, but to decrease the aromatic amino acid decarboxylase activity substantially (FONNUM *et al.*, 1977). These results have been confirmed, but in addition we found that the high affinity glutamate uptake decreased by 40–50% after this lesion, while the high affinity GABA uptake was unchanged.

Glutamergic afferents. The possibility of a glutamergic innervation of the nucleus accumbens was therefore studied in more detail. Firstly, a topographical distribution study of glutamate uptake demonstrated great differences in uptake activity in different regions, e.g. a high uptake was found in the neostriatum, slightly less in the lateral septum and nucleus accumbens, lower still in the olfactory tubercle, and very low in the globus pallidus. Of these regions, both the neostriatum, the lateral septum and the olfactory tubercle have been suggested to receive glutamergic fibers (DIVAC *et al.*, 1977; KIM, HASSLER, HAUG & PAIK, 1977; FONNUM & WALAAS, 1978; HARVEY, SCHOLFIELD, GRAHAM & APRISON, 1975). Secondly, subcellular fractionation of homogenates from the nucleus accumbens showed that glutamate uptake was most active in the synaptosome-enriched fraction. Thirdly, the time course of the decrease in uptake after placement of a lesion was generally comparable to that usually found for other transmitter markers following axotomy (REIS, GILAD, PICKEL & JOH, 1978). Also, the decrease in uptake was clearly not a result of changes in amino acid metabolism in the nucleus following lesions, as pilot experiments with the metabolically inert ligand D-aspartate, which travels by the same uptake mechanism as L-glutamate and L-aspartate (DAVIES & JOHNSTON, 1976), demonstrated approximately the same decrease after lesion (35–40% in three animals).

These results thus indicated that almost 50% of the high affinity glutamate uptake in the nucleus accumbens was specifically localized in nerve ter-

minals of an ascending projection to the nucleus. More restricted lesions were then performed to find the origin of this projection.

No significant difference in glutamate uptake was demonstrated after removal of the cingular or the pyriform cortex. Electrocoagulation of intralaminar thalamic nuclei also left the uptake unchanged. Transection of the medial forebrain bundle destroyed the aminergic innervation but had no effect on the uptake of glutamate. Thus fibers from the ventral tegmental area and brain stem, thalamus, and cingular or pyriform cortex did not possess a detectable part of this uptake capacity. Bilateral lesion of the fimbria/fornix, however, led to a decrease in glutamate uptake comparable to that found after hemitransection. Lesions designed to differentiate between the fibers cut by this lesion indicated that the glutamate-accumulating fibers innervating the nucleus accumbens through the fornix originate in regions located medial and caudal in the hippocampal formation. The excitatory fibers in the fimbria/fornix (De France & Yoshihara, 1975) which probably come from the subiculum and terminate in the nucleus accumbens (Swanson & Cowan, 1977) seem to fit this description reasonably well. Also, finding the same decrease in uptake after hemitransection and bilateral lesion of the fornix indicated that the projection was unilateral, as has been shown to be the case for the subicular fibers (Swanson & Cowan, 1977). Amino acid analysis after placement of the lesion demonstrated a significant decrease in the level of glutamate but not in aspartate. Thus, the subiculum–accumbens fibers are excitatory, they possess high affinity glutamate uptake, and have a high concentration of glutamate in their terminals. We therefore propose that these fibers are glutamergic.

Transmitters in local neurons

With the introduction of the selective neurotoxic compound kainic acid, which in several brain regions has been shown to destroy local neurons but to leave afferent fibers intact (Olney, Rhee & Ho, 1974; McGeer & McGeer, 1976; Coyle & Schwarcz, 1976; Hattori & McGeer, 1977; Herndon & Coyle, 1977; Fonnum & Walaas, 1978), a direct lesioning technique has been made available for analysis of the transmitter composition of local neurons. However, microscopical control of the type and the extent of the lesion will be necessary. In the present study, the lesion exhibited the same light-microscopic characteristics as those described by Schwarcz & Coyle (1977) in the neostriatum, with a selective loss of neuronal cells. However, the changes were also found to be present in adjoining regions. Thus, short projections to the nucleus accumbens (Powell & Leman, 1976) would probably also be affected. However, such projections are probably quantitatively insignificant (Swanson & Cowan, 1975), and we therefore think that the results of kainic acid injection may be interpreted mainly as a consequence of the loss of intrinsic cells.

Following injection of kainic acid, massive losses in both glutamate decarboxylase and ChAT activities were found. Indeed, in some animals the nucleus was found to be almost totally devoid of these enzymes. Acetylcholine and GABA therefore seem to be present almost exclusively in intrinsic neurons. GABA uptake decreased slightly less than the fall in glutamate decarboxylase activity and the activity of AChE decreased markedly less than that of ChAT. A small part of the GABA uptake is therefore probably localized outside kainic acid-sensitive neurons, possibly in glial cells, while a substantial part of the AChE activity could be present in glial cells (Hemminiki, Hemminiki & Giacobini, 1973) or in afferent non-cholinergic fibers (Lehmann & Fibiger, 1978). The aminergic marker aromatic amino acid decarboxylase was unaffected by kainic acid, demonstrating that the ascending dopaminergic and serotonergic fibers were intact.

In contrast, the high affinity uptake of glutamate was decreased by 40–50% after injection of kainic acid, the same decrease as found after lesion of the fornix. This result would seem to indicate either the existence of intrinsic glutamate/aspartate neurons in the nucleus accumbens, or alternatively that the afferent glutamergic fibers had been destroyed by kainic acid. To test these possibilities, we combined the lesion of the fornix and the injection of kainic acid, and found that the decreases in glutamate uptake were additive. Thus, the afferent fibers were not lesioned by kainic acid, and destruction of some local cells, either neurons or glia, which also can accumulate glutamate in a high affinity manner (Henn, Goldstein & Hamberger, 1974), must have been responsible. Since glial cells are left unchanged morphologically after the application of kainic acid (Olney *et al.*, 1974; Hattori & McGeer, 1977; Schwarcz & Coyle, 1977), the decrease in glutamate uptake probably reflects a degeneration of intrinsic glutamergic or aspartergic neurons.

Conclusions

Our results indicate the existence of a subiculum–accumbens projection which probably uses glutamate as transmitter, ascending aminergic fibers from the mesencephalon, and populations of intrinsic cholinergic, GABAergic and glutamergic or aspartergic cells.

The identification of these systems should help in clarifying the functional interplay of the different transmitter systems present in the nucleus, and also the functional organization of the different 'limbic' and 'extrapyramidal' regions connected with the nucleus accumbens.

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BIOCHEMICAL EVIDENCE FOR OVERLAPPING NEOCORTICAL AND ALLOCORTICAL GLUTAMATE PROJECTIONS TO THE NUCLEUS ACCUMBENS AND ROSTRAL CAUDATOPUTAMEN IN THE RAT BRAIN

IVAR WALAAS[1]
Norwegian Defence Research Establishment, Division for Toxicology, N-2007 Kjeller, Norway

Abstract The high affinity uptake of L-glutamate has been used to investigate the origin and distribution of putative glutamate fibers in restricted parts of the rostral caudatoputamen and the nucleus accumbens of the rat brain. Ablation of the frontal cortex reduced the glutamate uptake heavily (−77%) in the dorsal part of the ipsilateral caudatoputamen, but also led to significant decreases in the ventral parts of the ipsilateral caudatoputamen (−62% and −53%), in the ipsilateral nucleus accumbens (−25% and −18%) and in the contralateral dorsal part of the caudatoputamen (−21%). Lesion of the caudal neocortex reduced the glutamate uptake in the dorsal part of the ipsilateral caudatoputamen only (−23%). Lesions of the fimbria fornix reduced the glutamate uptake in the [illegible]s of the ipsilateral nucleus accumbens (−46% and −34%) and by approximately 20% in the whole dorsoventral extent of the anterior caudatoputamen.

The results indicate that the frontal neocortex distributes fibers which may use glutamate as neurotransmitter both to the whole ipsilateral caudatoputamen and to the nucleus accumbens, and also to the dorsal parts of the contralateral caudatoputamen. The caudal neocortex probably sends such fibers to the dorsal ipsilateral caudatoputamen, and the caudal allocortex sends such fibers through the fimbria/fornix to the nucleus accumbens and the ventral part of the ipsilateral caudatoputamen. The results thus corroborate previous suggestions of close similarities between the nucleus accumbens and the ventral caudatoputamen.

RECENT anatomical and neurochemical evidence indicates that the nucleus accumbens septi possibly might be regarded as an integral part of the rostral neostriatum (HEIMER & WILSON, 1975; SWANSON & COWAN, 1975; FONNUM, WALAAS & IVERSEN, 1977; NAUTA, SMITH, FAULL & DOMESICK, 1978; WALAAS & FONNUM, 1979*a*). Both these nuclei receive major cortical projections, and anatomical investigations have shown that the neostriatum (caudatoputamen) receives most of these fibers from the neocortex (KÜNZLE, 1975; HEDREEN, 1977), while the nucleus accumbens receives most of its cortical input from the allocortical hippocampal formation (HEIMER & WILSON, 1975; SWANSON & COWAN, 1977). We have previously demonstrated that these two projections contain fibers which probably use glutamic acid as neurotransmitter (DIVAC, FONNUM & STORM-MATHISEN, 1977; WALAAS & FONNUM, 1979*a*), results which have been confirmed by other workers (MCGEER, MCGEER, SCHERER & SINGH, 1977; ZACZEK, HEDREEN & COYLE, 1979). The present study was designed to further investigate some aspects of the distribution pattern of these glutamate fibres, and in particular to study whether they terminate in separate fields or whether they overlap. The high affinity uptake of glutamate present in these fibres has therefore been analysed in the dorsal, middle or ventral parts of the rostral caudatoputamen and in the medial and lateral nucleus accumbens after separate or combined lesions of the afferent cortical projections. The effects on the cholinergic marker enzyme choline acetyltransferase (EC 2.3.1.6) and the monoamine marker enzyme aromatic amino acid decarboxylase (EC 4.1.1.26) (FONNUM *et al.*, 1977) have also been investigated, in order to control the density of local neurons and mesencephalic afferents after the different lesions. Some preliminary results have been reported (WALAAS & FONNUM, 1979*b*; WALAAS, 1980).

EXPERIMENTAL PROCEDURES

Male Wistar rats, 180–200 g body weight, were subjected to 4 types of lesions under anesthesia with fentanyl/fluanisone (Hypnorm vet., Mekos) and Valium (Roche). The frontal neocortex anterior to the bregma was removed unilaterally by suction down to the white matter under visual guidance, with the lesion extending ventrally to the rhinal fissure and the olfactory bulb (Fig. 1). In some of these animals a small part of the cortex anteriomedial to the forceps minor of the corpus callosum was spared (Fig. 2). The fibers from the hippocampal formation were lesioned either by a unilateral or bilateral knife cut through the fimbria and fornix 1 mm behind the bregma and 6 mm deep to the scull surface (Fig. 1) (WALAAS & FONNUM, 1979*a*), by a unilateral suction lesion through the dorsal parietal cortex which destroyed the septal part of the hippocampus and the fimbria under visual guidance, or by a hemitransection of the brain at the level of the globus pal-

[1] Present address: Department of Pharmacology, Yale University School of Medicine, New Haven CT 06510, U.S.A.

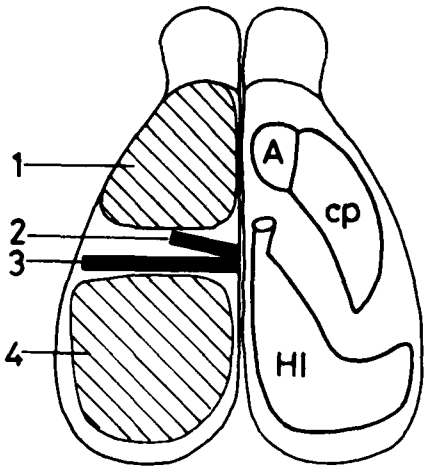

FIG. 1. Schematic outline of lesions of the corticofugal projections, viewed from above. 1, anterior decortication; 2, fornix transection; 3, hemitransection; 4, posterior decortication. *Abbreviations*: A, nucleus accumbens; cp, caudatoputamen; HI, hippocampus.

lidus (WALAAS & FONNUM, 1979*a*). A third group of animals received both a frontal decortication and a bilateral fornix cut and a fourth group received a suction lesion of the caudal neocortex and corpus callosum overlying the dorsal hippocampus (Fig. 1). The latter lesion was designed to control for possible neocortical fibres being destroyed by the fimbria fornix denervations.

Six to nine days after surgery, the animals were decapitated and the nucleus accumbens and the anterior caudatoputamen were dissected and divided as shown in Fig. 2 from frontal brain slices. The samples were homogenized in cold 0.32 M sucrose, pH 7.4, in glass-Teflon homogenizers to preserve nerve terminals (WALAAS & FONNUM, 1979*a*), and preincubated in Krebs-tris buffer in a shaking water bath at 25 C for 15 min. L-(2,3-^{3}H) glutamate (22.5 Ci mmol, New England Nuclear, Boston) was added (final conc. 10^{-7} M), the incubation was terminated after 3 min by rapid filtration, and the amount of labelled glutamate accumulated in the tissue was measured by liquid scintillation counting (FONNUM *et al.*, 1977).

For enzyme analysis, the homogenates were treated with Triton *X*-100 (final conc. 0.2% v/v) to release maximal activity, and choline acetyltransferase and aromatic amino acid decarboxylase were analysed with previously described methods (BROCH & FONNUM, 1972; FONNUM, 1975). All results were related to protein content (LOWRY, ROSEBROUGH, FARR & RANDALL, 1951), with bovine serum albumine used as standard.

RESULTS

In unoperated animals, the highest density of glutamate-accumulating structures was found in the ventral part of the rostral caudatoputamen, with distinctly lower uptake activity in the medial nucleus accumbens (Table 1). In contrast, cholinergic terminals as demonstrated by choline acetyltransferase activity were concentrated in the dorsal caudatoputamen and medial nucleus accumbens, while the amin-

TABLE 1. HIGH AFFINITY GLUTAMATE UPTAKE IN NUCLEUS ACCUMBENS AND CAUDATOPUTAMEN FOLLOWING LESIONS OF FIBERS FROM NEOCORTEX OR ALLOCORTEX

Region	Unoperated brain pmol 3 min mg protein	Frontal neocortical lesion per cent of unoperated		Caudal neocortical lesion per cent of unoperated		Fimbria fornix lesion or hemitransection per cent of unoperated		Bilateral fornix lesion and right frontal decortication per cent of unoperated	
		Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Right side	Left side
Nucleus accumbens Medial part	167 ± 23(9)	82 ± 9(10)**	112 ± 6(10)	119 ± 9(6)	100 ± 10(4)	54 ± 5(6)***	103 ± 7(5)	41 ± 5(4)**	59 ± 8(4)*
Lateral part	183 ± 10(8)	75 ± 6(10)***	108 ± 7(10)	119 ± 11(6)	105 ± 8(4)	66 ± 6(10)***	107 ± 5(9)	40 ± 5(4)**	71 ± 4(4)*
Caudatoputamen Ventral part	283 ± 22(8)	47 ± 4(15)***	90 ± 4(15)	111 ± 8(7)	107 ± 9(4)	76 ± 9(9)**	101 ± 6(8)	31 ± 7(4)**	80 ± 6(4)*
Central part	273 ± 19(11)	38 ± 4(15)***	88 ± 3(15)	87 ± 5(11)	92 ± 11(4)	79 ± 6(14)*	99 ± 6(16)	21 ± 4(4)**	67 ± 4(4)*
Dorsal part	252 ± 18(6)	23 ± 2(6)**	79 ± 5(6)*	77 ± 9(6)*	85 ± 8(4)	82 ± 8(5)	97 ± 10(5)	14 ± 3(4)**	68 ± 2(5)*

Results presented as mean ± SEM (number of animals). Statistical significance assessed with Wilcoxon's two sample test. *$P < 0.05$, **$P < 0.02$, ***$P < 0.001$, compared with unoperated brain.

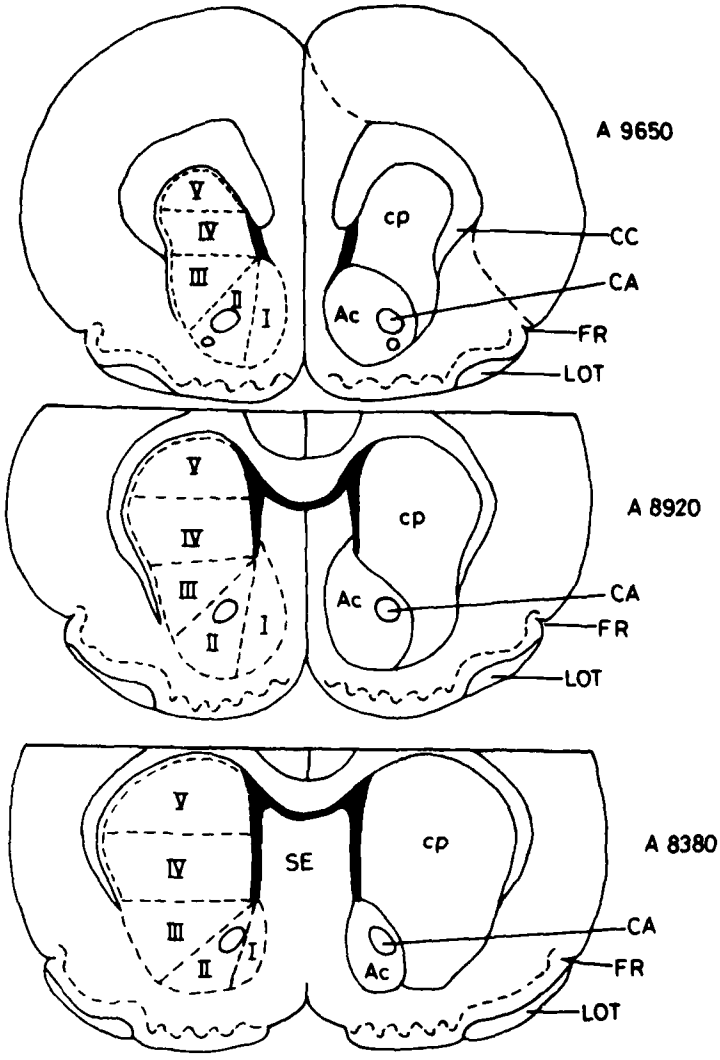

FIG. 2. Schematic drawing of frontal brain sections modified from KÖNIG & KLIPPEL (1963). Outline of dissected samples indicated on the left side. I, medial nucleus accumbens; II, lateral nucleus accumbens; III, ventral caudatoputamen; IV, central caudatoputamen; V, dorsal caudatoputamen. *Abbreviations*: Ac, nucleus accumbens; cp, caudatoputamen; CC, corpus callosum; CA, commissura anterior; FR, rhinal fissure; LOT, lateral olfactory tract; SE, septum. Numbers indicate distance in front of interaural line in μm. Outline of anterior neocortical lesion indicated on upper panel, right side.

ergic terminals containing aromatic amino acid decarboxylase were more evenly distributed (Table 2).

The denervation procedures led to significant effects on the glutamate uptake in all regions (Table 1). Unilateral rostral neocortical ablation consistently removed all cortical tissue on the frontal dorsolateral part of the hemisphere, but usually left a small part of the cingular, suprarhinal and anteromedial cortices intact. This lesion reduced the glutamate uptake significantly in all ipsilateral regions, most heavily in the dorsal caudatoputamen (−77%) and least in the medial accumbens (−18%). Contralaterally, the glutamate uptake decreased significantly in the dorsal caudatoputamen (−21%) and also slightly in the central sample (−12%, not significant). Second, ablation of the caudal dorsal neocortex and corpus callosum overlying the dorsal hippocampus decreased the glutamate uptake significantly in the ipsilateral dorsal caudatoputamen (−23%). Third, unilateral destruction of the allocortical fibres in the fimbria/fornix by means of three different procedures all reduced the glutamate uptake in both parts of the ipsilateral nucleus accumbens (−45% and −35%) and in the ipsilateral ventral and central parts of the caudatoputamen (−24% and −21%). Two of these procedures, i.e., the brain hemitransections and the fornix transections, also destroyed afferents to the nucleus accumbens running in the stria terminalis, while no

TABLE 2. AROMATIC AMINO ACID DECARBOXYLASE AND CHOLINE ACETYLTRANSFERASE IN NUCLEUS ACCUMBENS AND CAUDATOPUTAMEN FOLLOWING CORTICAL LESIONS, FORNIX LESIONS OR HEMITRANSECTIONS

Region		Unoperated brain μmol/h/g protein	Ipsilateral frontal decortication, per cent of unoperated	Fornix lesion, per cent of unoperated	Hemitransection, per cent of unoperated
Nucleus accumbens	AAD	49.6 ± 3.5 (6)	103 ± 5 (5)	106 ± 10 (11)	23 ± 5 (4)*
Medial part	ChAT	216 ± 33 (5)	96 ± 3 (6)	91 ± 3 (5)	—
Lateral part	AAD	47.9 ± 4.4 (6)	91 ± 9 (5)	104 ± 11 (11)	12 ± 1 (4)*
	ChAT	157 ± 19 (5)	91 ± 3 (6)	83 ± 13 (5)	104 ± 11 (4)
Caudatoputamen	AAD	58.9 ± 3.3 (6)	95 ± 7 (5)	94 ± 10 (11)	13 ± 1 (4)*
Ventral part	ChAT	172 ± 5 (10)	96 ± 6 (5)	109 ± 7 (5)	116 ± 13 (4)
Central part	AAD	54.5 ± 3.6 (6)	101 ± 9 (5)	94 ± 12 (6)	18 ± 3 (4)*
	ChAT	232 ± 9 (10)	91 ± 5 (5)	98 ± 7 (5)	110 ± 10 (4)
Dorsal part	AAD	56.1 ± 2.2 (5)	88 ± 3 (5)		17 ± 5 (4)*
	ChAT	255 ± 33 (5)	86 ± 6 (5)	98 ± 3 (5)	—

Results presented as mean ± SEM (number of animals). *$P < 0.01$ (Wilcoxon's 2 sample test).
Abbreviations: AAD, aromatic amino acid decarboxylase; ChAT, choline acetyltransferase.

visible damage could be seen in this fiber bundle after the suction lesions. After all these operations, the dorsal caudatoputamen displayed approximately the same reduction in uptake activity as after a caudal neocortical lesion. No significant effects were found in any regions on the contralateral side after the caudal lesions (Table 1). More extensive lesions substantiated these findings. Combining a unilateral frontal decortication with a bilateral fornix lesion demonstrated additive effects on the glutamate uptake in all regions ipsilateral to the frontal cortex ablation (Table 1). Contralaterally, such additive effects were only found in the 2 dorsal parts of the caudatoputamen, confirming that only these parts of the rostral caudatoputamen receive inputs from the contralateral frontal cortex (Fig. 3). Analysis of the activities of choline acetyltransferase and aromatic amino acid decarboxylase revealed that the densities of cholininergic and aminergic terminals were unchanged following both neocortical and allocortical lesions in all regions studied (Table 2). In the hemitransected animals, the aromatic amino acid decarboxylase activity decreased by 75–88% in both caudatoputamen and nucleus accumbens. These results agree well with our previous studies (FONNUM *et al.*, 1977; WALAAS & FONNUM, 1979a).

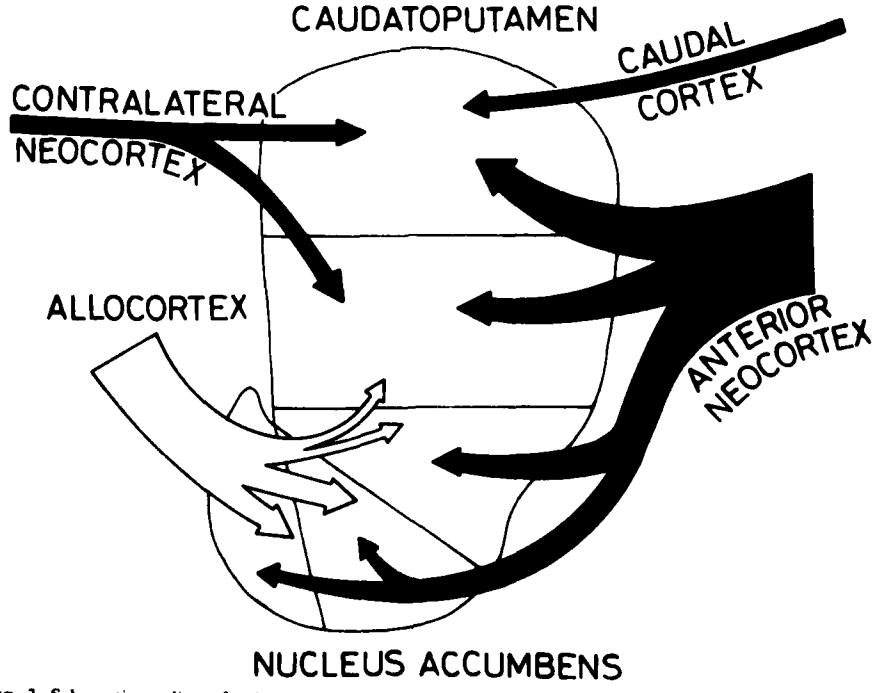

FIG. 3. Schematic outline of origin and distribution of putative glutamate-using cortex fibres to the caudatoputamen and nucleus accumbens in the rat brain.

DISCUSSION

The present study confirms that lesions of cortical neurons in the rat brain decrease the glutamate uptake capacity in the caudatoputamen and nucleus accumbens without destroying intrinsic cholinergic cells or aminergic fibres from the mesencephalon (DIVAC *et al.*, 1977; MCGEER *et al.*, 1977; WALAAS & FONNUM, 1979*a*). Thus, this glutamate uptake capacity is probably concentrated in the terminals of the cortico–striatal and cortico–accumbens fibres. It would therefore appear to be well suited as a biochemical 'marker' in studies on both the topography and terminal density in addition to investigations on the transmitter identity of these projections.

The nucleus accumbens and the ventral caudatoputamen

The major question investigated in the present work is whether the allocortical fibres to the nucleus accumbens distribute in a terminal field clearly separated from the fundus of the anterior caudatoputamen. Recent anatomical studies using either sensitive staining methods after anterograde degeneration (HEIMER & WILSON, 1975) or autoradiographic studies after anterograde transport of tritiated protein (SWANSON & COWAN, 1977) have clearly demonstrated the existence of these fibres, but not defined their terminal fields unequivocally. The present results appear to answer this question: those allocortical fibres coming through the fimbria/fornix and displaying glutamate uptake clearly extend outside the nucleus accumbens as usually defined (KÖNIG & KLIPPEL, 1963; NAUTA *et al.*, 1978). Furthermore, assuming that these allocortical fibres all have approximately the same glutamate uptake capacity, the results indicate that the terminal density of this projection probably is greatest in the medial nucleus accumbens (where these fibres are responsible for the uptake of approximately 75 pmol/mg protein/3 min of the labelled glutamate, calculated from Table 1. However, the density is only slightly lower in the lateral nucleus accumbens (approximately 65 pmol/mg protein/3 min) and in the ventral caudatoputamen (approximately 55 pmol/mg protein/3 min). Thus, no sharp boundary separates the nucleus accumbens and the ventral caudatoputamen in this respect.

The present study also demonstrates that the ipsilateral frontal neocortex distributes fibres to the nucleus accumbens, thus confirming recent anatomical studies (BECKSTEAD, 1979). The terminal density of this putative glutamate-using projection is more difficult to estimate, as some of the fibres may originate in regions in the anteriomedial cortex (BECKSTEAD, 1979) which partly may have survived the ablation procedure (Fig. 2). However, my results indicate that they probably distribute terminal boutons in the nucleus accumbens less densely than do the allocortical fibres (Table 1). In contrast, the ventral caudatoputamen lateral to the nucleus accumbens has a much higher density of neocortical than allocortical glutamate fibres (glutamate uptake: approximately 140 pmol/mg protein/3 min vs 55 pmol/mg protein/3 min, see above). Thus, the terminal field of the neocortical fibres in the caudatoputamen does exhibit some sort of boundary towards the nucleus accumbens, even if such fibres also penetrate into the accumbens proper.

In conclusion, therefore, the nucleus accumbens has the same qualitative pattern of cortical glutamate-fibre innervation as the ventral caudatoputamen, but the allocortical input dominates quantitatively in the nucleus accumbens while the neocortical input dominates in the ventral caudatoputamen. The distribution of cholinergic terminals found in this study also does not distinguish the nucleus accumbens clearly from the caudatoputamen, and the pattern of afferent dopaminergic innervation and acetylcholinesterase staining is also very similar (FALLON & MOORE, 1978; LINDVALL & BJÖRKLUND, 1978; FONNUM *et al.*, 1977). However, the detailed distribution of catecholamines is different, with highest concentration of noradrenaline found in the medial accumbens and negligible amounts in the cadatoputamen (OPSTAD & WALAAS, unpublished). The two nuclei also exhibit differences in the density and projection pattern of γ-aminobutyrate neurons (FONNUM *et al.*, 1977; WALAAS & FONNUM, 1979*c*, 1980). Therefore, despite the anatomical similarities between the 2 nuclei (HEIMER & WILSON, 1975; HEIMER, 1978; NAUTA *et al.*, 1978), the bulk of the neurochemical evidence indicates that the nucleus accumbens probably should be regarded as a distinct part of the 'ventral striatum' (HEIMER & WILSON, 1975; HEIMER, 1978) and not as an integral part of the caudatoputamen.

The dorsal caudatoputamen

Two aspects of the glutamate-projection to the caudatoputamen should also be briefly mentioned. First, the dorsal part of the caudatoputamen appears to receive an input of such fibres from the contralateral frontal neocortex. This confirms previous results from this laboratory (DIVAC *et al.*, 1977), and is in agreement with anatomical studies in other species (CARMAN, COWAN, POWELL & WEBSTER, 1965; KÜNZLE, 1975). Second, a significant part of the glutamate uptake in the dorsal caudatoputamen appears to originate in the caudal neocortex. These fibres were not found when the whole caudatoputamen was tested (FONNUM, STORM-MATHISEN & DIVAC, 1981) and probably represents a restricted input to the dorsal caudatoputamen only. This interesting projection might possibly be involved in transferring information from the visual cortex to the basal ganglia (HOLLÄNDER, TIETZE & DISTEL, 1979). Lastly, the dorsal striatal regions had considerably less residual glutamate uptake activity than the ventral samples after combined cortical denervations (30–40 pmol/mg protein/3 min vs 80–100 pmol/mg protein/3 min, respectively, calculated from Table 1). These ventral regions may therefore contain more dense populations of

either local glutamate neurons, glial cells accumulating glutamate (HENN, GOLDSTEIN & HAMBERGER, 1974) or glutamate fibres arriving from more ventral cortical regions (HEIMER & WILSON, 1975). In the nucleus accumbens, injections of the selective neurotoxic compound kainic acid, which probably destroys local neurons but leaves afferent fibres and glial cells intact, decreased the glutamate uptake significantly (WALAAS & FONNUM, 1979*a*). Thus, this nucleus may contain at least some local glutamate (or aspartate-using) neurons. However, some cortical neurons may also have been destroyed by this lesion (FRIEDLE, KELLY & MOORE, 1978; WURTHELE, LOVELL, JONES & MOORE, 1978). The origin of this glutamate uptake will therefore require further study.

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THE EFFECT OF INTRAHIPPOCAMPAL KAINIC ACID INJECTIONS AND SURGICAL LESIONS ON NEUROTRANSMITTERS IN HIPPOCAMPUS AND SEPTUM

F. Fonnum and I. Walaas

Norwegian Defence Research Establishment, Division for Toxicology
P.O. Box 25, 2007 Kjeller, Norway

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Abstract—Local injection of kainic acid (2 μg) was accompanied by destruction of intrinsic neurons in the dorsal part of hippocampus. The lesion was accompanied by a 25% reduction in glutamate decarboxylase activity, a 60% reduction in the high affinity uptake of L-glutamate, a 40–60% reduction in the endogeneous levels of aspartate, glutamate and GABA and no changes in the activities of choline acetyltransferase or aromatic amino acid decarboxylase in the dorsal hippocampus. Unilateral destruction of neurons in the dorsal hippocampus was followed by a 20–40% reduction in the high affinity uptake of glutamate in lateral, but not in medial septum, on both sides. There was no reduction in choline acetyltransferase, glutamate decarboxylase or aromatic amino acid decarboxylase activities in the lateral or medial part of the septum. Transection of fimbria and superior fornix was accompanied by a severe reduction in choline acetyltransferase and aromatic amino acid decarboxylase activity in hippocampus, in the high affinity uptake of glutamate and in the endogenous level of glutamate in the lateral septum. The results are consistent with the concept that in the hippocampus kainic acid destroys intrinsic neurons and not afferent fibres. It seems therefore that all GABAergic fibres in the hippocampus belong to intrinsic neurons whereas glutamergic and aspartergic neurons belong partly to local neurons. The connection from the hippocampus to the lateral septum probably uses glutamate as a transmitter.

Hippocampus including the area dentata is a relatively simple laminated structure with restricted afferent fibres and few intrinsic cell types. In recent work the localization of putative neurotransmitters in afferent fibres has been well documented. The cholinergic structures are distributed in laminae (Fonnum, 1970) and are derived from cells in the medial septum and nucleus of the diagonal band (Lewis *et al.*, 1967; Oderfeld-Nowak *et al.*, 1974). The serotonergic and noradrenergic fibres are of extrahippocampal origin (Storm-Mathisen & Guldberg, 1974), and are derived from cells in the median raphe nucleus (Lorens & Guldberg, 1974; Azmitia & Segal, in press) and locus coeruleus (Ross & Reis, 1974), respectively.

Recent experiments also indicate that glutamate and aspartate may function as transmitters in some afferent fibre systems in hippocampus, e.g. the perforant path and commissural fibres (Nadler *et al.*, 1976; Storm-Mathisen, 1977).

It is more difficult to obtain direct evidence to identify the neurotransmitters in intrinsic neurons. There is strong circumstantial evidence that GABA in hippocampus is present in local neurons (Storm-Mathisen, 1972) and also that glutamate or aspartate may be the transmitter of intrinsic systems such as the mossy fibres and the Schaffer collaterals (Crawford & Connor, 1973; Storm-Mathisen, 1977).

Recently it has been suggested that local injection of kainic acid, a powerful glutamate analogue, in other brain regions destroys intrinsic neurons but not afferent fibres (Olney *et al.*, 1974; Schwarcz & Coyle, 1977*a* & *b*; Hattori & McGeer, 1977; Walaas & Fonnum, 1978, in press). We have therefore studied the effect of kainic acid injection on the neurotransmitter candidates in hippocampus. Since the pyramidal cells in hippocampus project to the septum (Raisman *et al.*, 1966), we have compared the effect of kainic acid in dorsal hippocampus with surgical transection of the hippocampo-septal fibres on neurotransmitters in hippocampus and septum. There are only limited data on localization of neurotransmitters in septum (Fonnum, 1978).

The activities of choline acetyltransferase (ChAT, EC 2.3.1.6), glutamate decarboxylase (GAD, EC 4.1.1.15) and aromatic L-amino acid decarboxylase (AAD, EC 4.1.1.28) have been used as markers for cholinergic, GABAergic and catecholaminergic fibres respectively (Fonnum, 1975*a*), while the so-called high affinity uptake of L-glutamate has been used as a marker for glutamergic or aspartergic fibres (Young *et al.*, 1974; Storm-Mathisen, 1977; Divac *et al.*, 1977; Lund-Karlsen & Fonnum, 1978). Since L-glutamate and L-aspartate are taken up into synaptosomes by the same mechanism (Balcar & Johnston, 1972), amino acid analysis has been used to differen-

Abbreviations used: ChAT, choline acetyltransferase; GAD, glutamate decarboxylase; AAD, aromatic L-amino acid decarboxylase.

tiate between the effect on aspartergic and glutamergic neurons (Lund Karlsen & Fonnum, 1978).

MATERIALS AND METHODS

Albino male rats, weighing ca 180 g, were obtained from Dr Möllergaard-Hansens Avislaboratorium, Denmark. Kainic acid was obtained from Sigma Chem Co., St. Louis, U.S.A.

Treatment of animals

The animals were anesthetized with a mixture of diazepam–Hypnorm and placed in a stereotaxic apparatus. Kainic acid was dissolved in 0.9% NaCl (4 mg/ml) and the pH adjusted with NaOH to 7.2. Over a period of 1 min 0.5 μl was infused through a hole in the scull into the dorsal hippocampal region at coordinates A = +3.5, L = 2.5, D = +2.5 according to König & Klippel (1963). Sham-injected animals received the same amount of 0.9% NaCl. In pilot experiments, this was found to be without effect on the neurochemical markers analysed, and unlesioned animals were therefore routinely used as controls.

The fornix lesion was performed with a small scalpel 2 mm behind the bregma, lowered 6 mm from the scull surface and moved 4 mm laterally from the midline.

Preparation of tissue

The animals were decapitated after 5–7 days. The brains were carefully taken out and transverse slices (400 μm) prepared with a Sorvall tissue chopper. The injection site was inspected macroscopically, and the animal used if the injection was located within the middle third of the dorsal hippocampus. Samples for analysis were taken from slices within 1 mm from the injection site in the rostrocaudal direction while samples from ventral hippocampus in the injected animals were taken from the same or the adjacent slice.

For distribution studies, hippocampus from normal animals was dissected out, freed from subiculum and fimbria, and divided in three equal parts. The dorsal and ventral samples were analysed separately.

The samples from septum were taken from slices 8.6–7.2 mm in front of the interaural line and dissected according to König & Klippel (1963). The medial sample was taken dorsal to the level of the commissura anterior. It thus contained the medial septal nucleus, and a part of the nucleus of the diagonal band.

Biochemical methods

The samples were homogenized in 0.32 M-sucrose (2% w/v) in such a way as to preserve synaptosomes (Fonnum, 1975*a*).

The uptake of 10^{-4} M-L-glutamate was studied by incubating a 5 μl sample (10–15 μg protein) in 0.5 ml Tris Krebs medium for 3 min at 25 °C as previously described (Fonnum *et al.*, 1977). The uptake varied linearly with the weight of tissue under these conditions.

For enzyme assays the homogenate was treated with 0.2% (v/v, final conc.) of Triton X-100. ChAT was determined as described by Fonnum (1975*b*), AAD as described by Broch & Fonnum (1972) and GAD by the method of Albers & Brady (1959) as modified by Fonnum *et al.* (1977). Protein was determined by the method of Lowry *et al.* (1951).

For amino acid analysis the animals were killed by focussed microwave irradiation for 2.5 s (Guidotti *et al.*, 1974). The samples were dissected out and homogenized in 200 mM-bicarbonate acetone (1:1). Amino acid content in the supernatant was determined by the dansylation technique using [^{3}H]dansyl chloride and ^{14}C-labelled amino acids as internal standards as described by Lund Karlsen & Fonnum (1976). The dansyl amino acids were eluted with 0.75 ml acetone:acetic acid (3:2 v/v), and counted in a Packard scintillation counter Model 3380 fitted with an absolute activity analyser Model 544 set for simultaneous determination of ^{3}H and ^{14}C.

For histological staining, transverse sections (40 μm) were cut in a cryostat, allowed to dry on microscope slides and stained either with thionin or for acetylcholinesterase (AChE, EC 3.1.1.7) with ethopropazine as inhibitor for butyrylcholinesterase (Storm-Mathisen, 1970).

Statistical significance of difference between dorsal and ventral hippocampus, left and right hippocampus or left and right septum was assessed by the Wilcoxon paired comparison test. Comparison between lesioned and unlesioned animals was performed with the Wilcoxon two sample test (Hodges & Lehmann, 1964).

RESULTS

Previous work has shown that biochemical changes after fimbrial transection (Storm-Mathisen, 1972) and neuronal necrosis after kainic acid injections (Schwarcz & Coyle, 1977*a*, *b*) are fully developed within 2–5 days. We therefore limited our investigations to a study of the effect of these factors on biochemical parameters after 5–7 days. Five days after injection of kainic acid into the dorsal hippocampus a massive loss of pyramidal and granule cells was found. Figure 1 shows adjacent sections stained for cells and for AChE from an animal where the injection was confined to the lateral hippocampus. In regio inferior and lateral parts of regio superior and area dentata an almost complete loss of cells was found, while apparently normal neurons could be seen in the medial part. In sections from two other animals (not shown) a complete loss of pyramidal and granule cells was found. We assume that the more diffusely localized Golgi II and basket cells were probably also affected although this cannot be properly seen in our section. In contrast, the distribution of AChE staining did not change on the injected side. The laminated structure and intensity of the staining were well preserved (Fig. 1c).

Since our lesion was restricted to the dorsal hippocampus we were also interested in studying the effect this lesion had on the ventral hippocampus. Several of the parameters differed between the normal dorsal and ventral hippocampus. The activities of AAD and ChAT were found to be significantly higher in ventral hippocampus, while GAD and glutamate uptake were more evenly distributed (Table 1).

Kainic acid injection led to a 74% decrease in GAD activity and 63% decrease in glutamate uptake under high affinity conditions but left ChAT and AAD activities intact (Table 2). This lesion affected these parameters neither in the contralateral hippocampus (Table 2) nor in the ventral hippocampus on the in-

FIG. 1. A Thionine-stained 40 μm section from dorsal hippocampus injected with 2 μg kainic acid in the lateral part. B Thionine-stained section from contralateral hippocampus. C AChE-stained section from kainate-injected hippocampus. D AChE-stained section from contralateral hippocampus. Sections C and D are taken adjacent to sections A and B.

TABLE 1. DISTRIBUTION OF NEUROTRANSMITTER MARKERS IN HIPPOCAMPUS

	Dorsal hippocampus (μmol/h/g protein)	Ventral hippocampus (μmol/h/g protein)
Glu uptake	2.66 ± 0.23 (8)	2.45 ± 0.16 (8)
ChAT	57 ± 3 (8)	74 ± 3 (8)*
GAD	185 ± 16 (6)	206 ± 21 (6)
AAD	3.5 ± 0.2 (6)	5.6 ± 0.3 (6)*

The results represent mean ± S.E.M. (number of animals).
* $P < 0.02$.

TABLE 2. THE EFFECT OF INTRAHIPPOCAMPAL INJECTION OF KAINIC ACID ON NEUROTRANSMITTER MARKERS IN HIPPOCAMPUS

	Unoperated (μmol/h/g protein)	Uninjected side (% of unoperated)	Injected (% of unoperated)
Glu uptake	2.67 ± 0.18 (6)	93 ± 8 (7)	37 ± 4 (7)*
GAD	192 ± 14 (10)	102 ± (7)	26 ± 4 (7)*
ChAT	62 ± 6 (6)	101 ± 6 (7)	90 ± 7 (7)
AAD	3.7 ± 0.2 (6)	103 ± 2 (5)	97 ± 3 (5)

The results represent mean ± S.E.M. (number of animals).
* $P < 0.01$ compared to unoperated animals.

jected side (ChAT: 93 ± 12%, GAD: 85 ± 7%, AAD: 92 ± 12% and glutamate uptake: 80 ± 15%, results expressed as mean ± S.E.M. and are in percent of activities in ventral hippocampus on uninjected side). Amino acid analysis showed that the endogenous levels of aspartate, glutamate and GABA were significantly reduced, while alanine, glycine and glutamine were unchanged (Table 3).

In the septum, the glutamate uptake under high affinity conditions and GAD activity were concentrated in the lateral part, the ChAT activity was concentrated in the medial part, while AAD activity was evenly distributed (Table 4). Following kainic acid injection into hippocampus, the glutamate uptake was significantly reduced in the lateral septum on both the ipsilateral side (35% decrease) and the contralateral side (23% decrease). The other neuronal markers were unaffected by this lesion both in lateral and medial septum (Table 4).

The effects of destruction of cells in hippocampus by kainic acid injection was compared with the effect of cutting the afferents and efferents by surgical transection of fornix/fimbria. Following fornix/fimbria lesion, ChAT activity was decreased by 90% and AAD activity was decreased by 45% in hippocampus (Table 5). GAD activity and high affinity glutamate uptake were unchanged by this lesion (Table 5). In the medial septum, the fornix/fimbria lesion was without effect on the neuronal markers (Table 5). In the lateral septum, a unilateral fornix lesion led to a 40% decrease in glutamate uptake on the ipsilateral side, while a bilateral lesion led to a 70% decrease in glutamate uptake (Table 5). This lesion led to a slight but significant increase in ChAT activity in the lateral but not medial septum, while GAD and AAD activities were unchanged (Table 5). Amino acid analysis demonstrated a high glutamate concentration in lateral septum of unoperated animals (Table 6). A bilateral fornix lesion led to a significant decrease in the concentration of glutamate, but not of aspartate, glycine, GABA or glutamine (Table 6).

DISCUSSION

The concept that kainic acid, the toxic glutamate analogue, destroys cell bodies and not afferent fibres receives strong support in this study. Thus ChAT and AAD in the dorsal hippocampus were unaffected by kainic acid injection (Table 2), but were markedly decreased after fimbria/fornix transection (LEWIS *et al.*, 1967; Table 4). On the other hand, GAD which did not decrease after a series of lesions of afferents to hippocampus (STORM-MATHISEN, 1972), was almost completely lost after kainic acid injection (Table 2). Glutamergic/aspartergic fibres which have been suggested to be partly of extrahippocampal origin (STORM-MATHISEN, 1977; NADLER *et al.*, 1976) were partly destroyed (Tables 2 and 3).

TABLE 3. AMINO ACIDS IN HIPPOCAMPUS AFTER INTRAHIPPOCAMPAL INJECTION OF KAINIC ACID

	Uninjected side (pmol/μg protein)	Injected side (% of uninjected)
Glu	92.1 ± 10.3	63 ± 7*
Asp	14.0 ± 2.0	59 ± 8*
Gly	10.3 ± 2.1	76 ± 10
GABA	25.3 ± 3.4	51 ± 9†
Gln	46.3 ± 6.3	83 ± 15
Ala	7.3 ± 1.5	82 ± 5

Results represent mean ± S.E.M. from 6 animals.
* $P < 0.05$
† $P < 0.03$

Table 4. The effect of intrahippocampal injection of kainic acid on neurotransmitter markers in septum

	Lateral septum		
	Unoperated (μmol h g protein)	Uninjected side (% of unoperated)	Injected side
Glu uptake	2·66 ± 0·13 (8)	77 ± 8 (5)*	65 ± 6 (6)*
GAD	262 ± 15 (5)	120 ± 13 (4)	90 ± 8 (5)
ChAT	45 ± 2 (6)	94 ± 10 (5)	86 ± 10 (5)
AAD	7·3 ± 0·9 (5)	103 ± 15 (5)	85 ± 8 (5)
	Medial septum		
Glu uptake	0·95 ± 0·14 (4)	96 ± 8 (4)	86 ± 10 (4)
GAD	93 ± 8 (7)	116 ± 9 (4)	112 ± 7 (4)
ChAT	120 ± 16 (5)	113 ± 6 (4)	105 ± 7 (5)
AAD	7·4 ± 0·4 (5)	80 ± 17 (5)	97 ± 2 (5)

The results represent mean ± S.E.M. (number of animals).
* $P < 0.01$ compared to unoperated animals.

Table 5. The effect of fornix fimbria lesion on neurotransmitter markers in hippocampus and septum

		Hippocampus	Medial septum (% of unoperated controls)	Lateral septum
Glu uptake	bilat. lesion	108 ± 8 (6)	91 ± 9 (5)	30 ± 4 (6)*
	unilat. lesion	106 ± 4 (5)	88 ± 11 (5)	59 ± 5 (5)†
ChAT	bilat. lesion	8 ± 2 (5)*	103 ± 3 (5)	128 ± 10 (6)†
	unilat. lesion	16 ± 10 (5)*		
GAD	bilat. lesion	92 ± 11 (4)	87 ± 10 (4)	95 ± 10 (4)
AAD	bilat. lesion	55 ± 6 (4)†	87 ± 8 (4)	109 ± 11 (4)

The results represent mean ± S.E.M. (number of animals) and are expressed as % of unoperated controls simultaneously analysed.
* $P < 0.01$
† $P < 0.03$ compared to unoperated animals.

The findings on cholinergic structures in hippocampus deserve some special comments. In other regions which have been submitted to kainic acid injection, e.g. striatum (McGeer & McGeer, 1976, Schwarcz & Coyle, 1977*a*), retina (Schwarcz & Coyle, 1977*c*), nucleus accumbens (Walaas & Fonnum, in press), or in the mediobasal hypothalamus (Walaas & Fonnum, 1978) and retina (Lund Karlsen & Fonnum, 1976) of glutamate-treated animals, there has always been a dramatic decrease in ChAT. One could therefore be led to think that in cholinergic neurons the excitotoxic effects of kainic acid and glutamic acid (Olney *et al.*, 1974) were not restricted to cell bodies. The present results demonstrate that cholinergic axonal terminals are insensitive to kainic acid. The small decrease in ChAT (10% in Table 1) corresponded to the proportion of ChAT resistant to fimbrial fornix transection (8–16% in Table 5) and may be due to a small proportion of cholinergic cells with strong AChE staining in 'area 31' of the molecular layer in regio superior (Srebro & Mellgren, 1974). An additional finding in our investigation was the higher ChAT activity in the ventral than the dorsal part of hippocampus (Table 1). This agrees with the more intense AChE staining found in the ventral part (Malo *et al.*, 1977).

Table 6. Amino acids in lateral septum after fornix fimbria lesion

	Unlesioned brain (pmol/μg protein) n = 6	Lesioned brain (% of unlesioned) n = 5
Glu	12·1 ± 5·9	67 ± 3*
Asp	20·0 ± 1·6	91 ± 8
Gly	6·7 ± 0·3	96 ± 20
GABA	24·0 ± 1·4	82 ± 7
Gln	55·1 ± 5·2	116 ± 17

Results presented as mean ± S.E.M.
* $P = 0.002$

AAD which did not decrease after kainic acid injections, is present in serotonergic and noradrenergic fibres which are of extrahippocampal origin (Azmitia & Segal, 1978; Lindvall & Björklund, 1974). After transection of these afferent systems, however, a significant AAD activity was left in the hippocampus (Table 4. Storm-Mathisen & Guldberg, 1974). In cortex a substantial part of AAD has been suggested to be present in capillary walls (Kellogg *et al.*, 1973). The present results show that in hippocampus this presumed non-neuronal AAD activity is resistant to kainic acid.

Topographical studies of GAD activity in the hippocampal region have demonstrated that the enzyme activity is concentrated in the neuronal cell body layers and in the molecular layers (FONNUM & STORM-MATHISEN, 1969, STORM-MATHISEN & FONNUM, 1971). There is apparently no significant difference between dorsal and ventral parts (Table 1). The present work shows a large loss (Table 4) of GAD and GABA concomitant with the loss of hippocampal neurons after kainic acid. Since the loss of GAD was almost total in some cases, we assume that the activity in both cell body layers and molecular layers has been reduced. Taken together with the previously reported resistance of GAD to hippocampal deafferentation (STORM-MATHISEN, 1972), these results indicate that almost all GAD is present in intrinsic neurons in hippocampus.

The fall in high affinity uptake of glutamate and specific loss in endogenous glutamate and aspartate levels after kainic acid treatment are consistent with the possibility that intrinsic neurons could use glutamate or aspartate as transmitters. This has also been suggested after autoradiographic studies on the uptake of D-aspartate into hippocampal slices (TAXT *et al.*, 1977) and measurement of glutamate uptake into homogenates from different regions of hippocampus (STORM-MATHISEN, 1977), NADLER *et al.*, 1976). These studies together suggest that the putative glutamergic or aspartergic neurons which are destroyed by kainic acid could be the granule cells which give rise to the mossy fibres (BLACKSTAD & KJÆRHEIM, 1961), and the CA 3 pyramidal cells which give rise to the Schaffer collaterals (HJORT-SIMONSEN, 1973). Both cell types were seen to degenerate after intrahippocampal kainic acid injection (Fig. 1). The uptake of glutamate which remained after this treatment, could be due to the terminals of the perforant path which arises from cells in entorhinal cortex (NADLER *et al.*, 1976, STORM-MATHISEN, 1977). A part of the uptake may also be into glial elements (HENN *et al.*, 1974). It has also been suggested that the commissural fibres from the contralateral hippocampus use aspartate or glutamate as transmitters (STORM-MATHISEN, 1977, NADLER *et al.*, 1976). However, rostral deafferentation of hippocampus did not produce a significant loss in the glutamate uptake when the dorsal hippocampus was tested (Table 1, Table 4). Thus the commissural fibres in this part seem to represent only a small part of the total part of glutamergic aspartergic fibres.

The pyramidal cells in the hippocampal formation project through fimbria and fornix superior to the lateral part of the septum on both sides (RAISMAN *et al.*, 1966, SWANSON & COWAN, 1977). These fibres have been reported to be excitatory (DEFRANCE *et al.*, 1973, MCLENNAN & MILLER, 1974*a*). Our results suggest that glutamate could be the excitatory transmitter released from these fibres. Thus we found that high affinity glutamate uptake was concentrated in the lateral septum. Destruction of pyramidal cells in the dorsal hippocampus by kainic acid injection reduced the high affinity glutamate uptake in lateral septum, but not medial septum, on both sides. Similar results were obtained by unilateral fimbrial transection. As expected bilateral fimbria/fornix transection led to a more pronounced decrease. The effect on surgical transection were similar to the results obtained by STORM-MATHISEN & WOXEN OPSAHL (in press).

Since the reduction in glutamate uptake was only slightly higher after unilateral transection than after destruction of pyramidal cells in the dorsal part, we think that the dorsal hippocampus must be the most important source of these fibres. After bilateral transection of fimbria/fornix there was a significant loss of glutamate but not of the other amino acids including aspartate suggesting that the hippocampal–lateral septum connection uses glutamate as its transmitter.

The regional differences found for GAD and ChAT in septum are in general agreement with results obtained by the 'punching technique' (TAPPAZ *et al.*, 1976, PALKOVITS *et al.*, 1974). Thus cholinergic elements seem to be concentrated in the medial part, while GABAergic elements are concentrated in the lateral part (Table 4). The increase in ChAT activity in lateral septum after fornix/fimbria transection probably represents a 'pile up' of enzyme in the proximal parts of the severed axons which arise from medial septum as first described by LEWIS *et al.* (1967). Our results indicate that GAD in septum is resistant to caudal deafferentations, e.g. intrahippocampal kainic acid (Table 4), fornix transection (Table 5) or complete hemitransection through the globus pallidus (FONNUM *et al.*, 1977). Thus GABA seems to be present mainly in intrinsic neurons. This is in agreement with electrophysiological studies (MCLENNAN & MILLER, 1974*b*), which indicate that septum contains inhibitory interneurons. Some of these neurons could use GABA as transmitter.

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BIOCHEMICAL EVIDENCE FOR GLUTAMATE AS A TRANSMITTER IN HIPPOCAMPAL EFFERENTS TO THE BASAL FOREBRAIN AND HYPOTHALAMUS IN THE RAT BRAIN

I. Walaas and F. Fonnum
Norwegian Defence Research Establishment, Division for Toxicology,
Box 25, N-2007 Kjeller, Norway

Abstract The effects of bilateral transection of the fornix bundle on the high affinity uptake of glutamate and on the amino acid content in several nuclei of rat forebrain and hypothalamus were studied in order to investigate the possible role of glutamate as a transmitter of these fibres. This lesion decreased the high affinity uptake of L-glutamate by 60–70% in the mammillary body and lateral septum, and by 40–50% in the anterior diagonal band nucleus, the bed nucleus of the stria terminalis, the mediobasal hypothalamus and the nucleus accumbens. The content of endogenous glutamate in samples dissected from freeze-dried tissue also decreased significantly in these regions. Endogenous aspartate was slightly decreased in the anterior diagonal band nucleus and the mammillary body, but unchanged in the other regions. No significant changes were seen in the levels of serine, γ-aminobutyric acid, glutamine and taurine, except for an increase in glutamine and taurine in the bed nucleus of the stria terminalis. The high affinity uptake of γ-aminobutyric acid, tested in the bed nucleus of the stria terminalis, the mediobasal hypothalamus and the mammillary body, was unchanged after the lesion.

The results indicate that allocortical efferents innervating subcortical nuclei through the fornix might use glutamate as a transmitter. The study further supports the concept that glutamate plays an important role as transmitter of several different corticofugal fibre systems in mammalian brain.

Recent anatomical studies in rodent brain have demonstrated that most of the corticofugal fibres in the fornix bundle originate in the pyramidal cells in the subiculum and terminate ipsilaterally in the septum, basal forebrain nuclei and the hypothalamus (Swanson & Cowan, 1977; Meibach & Siegel, 1977). In addition, some fibres which distribute bilaterally to the lateral septum arise in the pyramidal cells in the CA3-CA1 regions of the hippocampus proper (Andersen, Bland & Dudar, 1973; Swanson & Cowan, 1977). The majority of both types of fornix fibres appear to release an excitatory transmitter, as found in both the septum (McLennan & Miller, 1974; DeFrance, Kitai & Shimono, 1973), the nucleus accumbens (DeFrance & Yoshihara, 1975) and the rostral part of the diagonal band nucleus (Hubbard, Mills & Sirett, 1979). In agreement with this, stimulation of the hippocampus or the subiculum increases the metabolic activity in the nuclei innervated by the fornix fibres (Kelot & Polfetti, 1979).

The identity of the neurotransmitter responsible for these effects has long remained unknown. However, we have recently demonstrated that destruction of hippocampal pyramidal cells or their axons in the fornix bundle selectively decreased both the high affinity uptake of glutamate and the content of endogenous glutamate in the lateral septum and the nucleus accumbens, thus indicating that this excitatory amino acid (Curtis, 1979; Curtis & Johnston, 1974) could be the transmitter released by these fibres (Fonnum & Walaas, 1978; Walaas & Fonnum, 1979*a*). Similar results have been obtained by other workers (Storm-Mathisen & Opsahl, 1978; Nitsch, Kim, Shimada & Okada, 1979; Zaczek, Hedreen & Coyle, 1979). Furthermore, release of D-[^{3}H]aspartate after specific stimulation of the hippocampo septal fibres also support the view that glutamate is the transmitter in the latter fibres (Malthe-Sörenssen, Skrede & Fonnum, 1980).

The present study has extended these observations. In an effort to identify the putative transmitter of the fibres in the fornix, the high affinity uptake mechanisms of glutamate and γ-aminobutyrate (GABA) and the concentration of several endogenous amino acids were analysed in some of the other target nuclei of the fornix bundle, in unoperated rat brains and in brains with a bilateral fornix transection. The regions analysed included the anterior part of the nucleus of the diagonal band, the bed nucleus of the stria terminalis, the mediobasal hypothalamus and the mammillary body, as well as the lateral septum and the nucleus accumbens. Some preliminary results have been presented (Fonnum, Lund-Karlsen, Malthe-Sörenssen, Skrede & Walaas, 1979).

EXPERIMENTAL PROCEDURES

Materials

L-[2,3-^{3}H]glutamic acid (22.5 Ci/mmol) was from New England Nuclear, Boston, and [U-^{14}C]-γ-aminobutyric

Abbreviation: GABA, γ-aminobutyrate

acid (GABA) (224 Ci/mol) was from the Radiochemical Centre, Amersham, England. Male Wistar rats, 180–210 g body wt, were from Möllergaard, Denmark. Other chemicals were commercially obtained.

Surgical operation

The rats were anaesthetized with diazepam and a mixture of fentanyl citrate and fluanisone (Hypnorm vet. Mekos; WALAAS & FONNUM, 1980), and placed in a stereotactic frame after KÖNIG & KLIPPEL (1963). A coronal slit was made in the skull with a dental drill 6.4 mm in front of the interaural line, and the fornix bundle was transected bilaterally with a small scalpel which was lowered 6 mm from the skull surface and moved 2 mm laterally from the midline (WALAAS & FONNUM, 1979*a*).

Sample analysis

For *uptake studies*, the operated animals together with unoperated controls were decapitated 7–10 days after lesion, and 0.4–0.6 mm frontal slices were prepared in the cold room with a Sorvall tissue chopper. The extent of the lesions were controlled visually, and the regions under study were dissected with razor blade splints under microscopic guidance (Fig. 1). The sample from the bed nucleus of the stria terminalis contained both the dorsal and ventral parts of the nucleus and also some tissue from the anterior commissure-bundle. The mediobasal hypothalamus sample, taken between the optic chiasm and the pituitary stalk contained the whole arcuate nucleus, the median eminence and a medial part of the ventromedial nucleus. The mammillary body was taken caudal to the origin of the pituitary stalk. The samples were homogenized (2% w/v) in cold 0.32 M sucrose, pH 7.4, with ten strokes in glass-Teflon homogenizers, and preincubated in buffer (FONNUM, WALAAS & IVERSEN, 1977) at 25°C for 10 min. [^{3}H]glutamate (final conc 10^{-7} M) and, in some experiments, [^{14}C]GABA (final conc 10^{-6} M) were added, and the incubation was stopped after 3 min by means of rapid filtration as described (FONNUM, STORM-MATHISEN & DIVAC, 1980). The amounts of labelled glutamate and GABA accumulated in the tissue were analysed by liquid scintillation spectrometry in a Packard Tricarb 3380 fitted with an absolute activity analyser set for simultaneous determination of [^{3}H] and [^{14}C]. Under the conditions used, uptake activity was proportional to amount of tissue (WALAAS & FONNUM, 1979*a*). The content of protein in the homogenates was analysed by the Lowry Method (LOWRY, ROSEBROUGH, FARR & RANDALL, 1951).

For *amino acid analysis*, operated and unlesioned animals were decapitated, and the brains rapidly removed and frozen on a microtome chuck with a CO_2-jet. Time interval between death and completion of the freezing was usually 1–1½ min. Frontal 40 μm sections from the tel- and diencephalon were then cut in a cryostat at −16°C, lyophilized and stored as described (FONNUM, STORM-MATHISEN & WALBERG, 1970). The samples were dissected under a stereomicroscope as indicated in Fig. 1, except for the sample from the bed nucleus of the stria terminalis, which consisted of the dorsal and posterior parts of the nucleus only. The dissected tissue from one nucleus was pooled into 2–5 samples and weighed on a Mettler microbalance (10–50 μg dry weight per pooled sample). The amino acids were extracted by homogenization in 2.5% trichloroacetic acid. In addition the homogenate was frozen and thawed once. Norleucine or DL-γ-aminoadipic acid was added as internal standards. Trichloroacetic acid was then extracted with water-saturated diethylether, and the samples were analysed in an automatic amino acid analyser (Kontron Liquimat III) as described (FONNUM *et al.*, 1980).

Comparison between operated and unlesioned animals was performed with the non-parametric Wilcoxon two-sample test.

RESULTS

Effect of lesions on high affinity uptake process for L-*glutamate*

In unlesioned brains, the capacity for high affinity uptake L-glutamate was concentrated in the lateral septum and nucleus accumbens, with lower activity found in the mammillary body, the bed nucleus of the stria terminalis, the anterior diagonal band nucleus

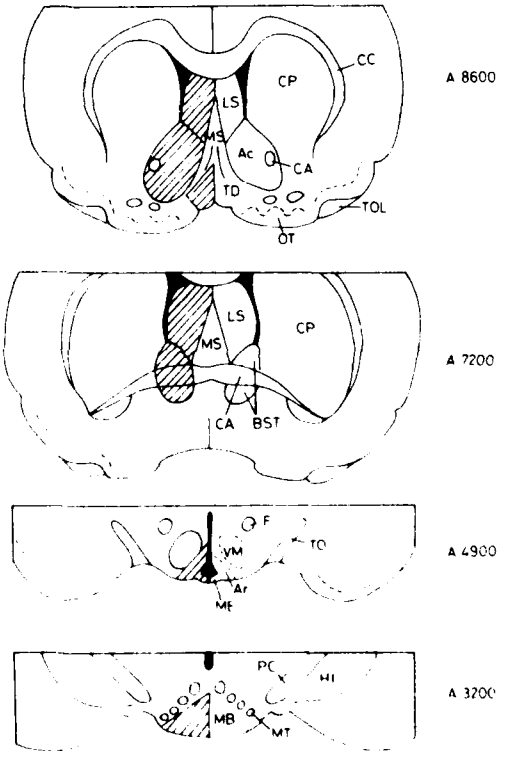

FIG. 1. Schematic drawing of coronal sections from rat brain, modified after KÖNIG & KLIPPEL (1963). Outline of dissected samples indicated on left side by hatched regions. On right side is approximate outline of brain nuclei. Numbers indicate distance in front of interneural line in μm. Abbreviations: Ac, nucleus accumbens; CA, anterior commissure; BST, bed nucleus of the stria terminalis; Ar, nucleus arcuatus; CC, corpus callosum; CP, caudatoputamen; F, fornix; HI, hippocampal formation; MS, medial septum; LS, lateral septum; OT, olfactory tubercle; TOL, lateral olfactory tract; TD, diagonal band nucleus; TO, tractus opticus; VM, nucleus ventromedialis hypothalami; ME, median eminence; MB, mammillary body; MT, mammillothalamic tract; PC, cerebral peduncle.

TABLE 1. HIGH AFFINITY AMINO ACID UPTAKE ACTIVITY IN RAT BRAIN SUCH FOLLOWING FORNIX TRANSECTION

	Glutamate uptake		GABA uptake	
	Unoperated brains pmole min mg protein	Operated brains % of unoperated	Unoperated brains nmole min mg protein	Operated brains % of unoperated
Anterior diagonal band nucleus	27.0 ± 4.8(8)	49 ± 6*(7)		
Nucleus accumbens	43.8 ± 1.9(5)	56 ± 4*(4)		
Lateral septum	51.3 ± 4.3(6)	39 ± 5*(12)		
Bed nucleus of the stria terminalis	31.5 ± 3.4(12)	56 ± 9*(12)	37.2 ± 5.1(5)	96 ± 15(4)
Mediobasal hypothalamus	22.8 ± 3.2(8)	57 ± 4*(10)	32.5 ± 5.1	82 ± 15(4)
Mammillary body	29.7 ± 5.9(5)	33 ± 9*(5)	29.1 ± 3.3(5)	90 ± 14(5)

Results presented as mean ± SEM (number of animals each assayed in triplicate).
Statistical significance: $*P < 0.02$.

and the mediobasal hypothalamus (Table 1). Amino acid analysis indicated that the content of both glutamate and glutamine was highest in the nucleus accumbens and lateral septum, followed by the dorsal part of the bed nucleus of the stria terminalis, the mediobasal hypothalamus, the mammillary body and the anterior diagonal band (Table 2). Aspartate was more evenly distributed. GABA was highly concentrated in the diagonal band nucleus and the lateral septum, followed by the nucleus accumbens and bed nucleus of the stria terminalis. However, these high values might be too high as a result of the high activity of the synthetic enzyme glutamate decarboxylase to be present in these nuclei (TAPPAZ, BROWNSTEIN & PAIKOWITZ, 1976; FONNUM *et al*., 1977; FONNUM & WALAAS, 1978). Considerable amounts of taurine and serine were also found in these regions (Table 2).

After bilateral fornix transection, the activity of the high affinity glutamate uptake decreased by 60–70% in the mammillary body and the lateral septum and by 40–50% in the nucleus accumbens, the anterior part of the diagonal band nucleus, the bed nucleus of the stria terminalis and in the mediobasal hypothalamus (Table 1). The uptake of GABA which was assayed simultaneously with the glutamate uptake in some experiments did not decrease significantly in any of the regions studied.

Effect of lesions on content of endogenous amino acids

The lesion also induced changes in the endogenous amino acids. Glutamate concentration decreased significantly in all regions, most heavily in the septum (−40%) and least pronounced in the diagonal band (−15%). Aspartate concentration decreased significantly in the diagonal band and mammillary body, but not in the septum, hypothalamus, nucleus accumbens and the bed nucleus of stria terminalis (Table 3). Glutamine and taurine concentration were significantly increased in the bed nucleus of stria terminalis, but unchanged in the other nuclei. Serine and GABA concentrations were unchanged in all regions (Table 3).

DISCUSSION

The results support the use of the high affinity uptake mechanism of L-glutamate and analysis of endogenous amino acid levels as means of the identifying nerve fibres using L-glutamate or L-aspartate as transmitters (YOUNG, OSTER-GRANITE, HERNDON & SNYDER, 1974; DIVAC, FONNUM & STORM-MATHISEN, 1977; LUND-KARLSEN & FONNUM, 1978; FONNUM & WALAAS, 1978; WALAAS & FONNUM, 1979*a*; FONNUM *et al*., 1980). Furthermore, they confirm our previous suggestion that glutamate is the main neurotransmitter in the neo-and allo-corticofugal fibres (FONNUM *et al*., 1979). Some further points also deserve comments.

Methodological considerations

Our previous studies have demonstrated that the amino acid uptake mechanisms present in crude sucrose homogenates predominantly represents neuronal uptake (review FONNUM *et al*., 1979). However, the uptake mechanism for L-glutamate does not distinguish between putative glutamate and aspartate-using fibres (BALCAR & JOHNSTON, 1972; LUND-KARLSEN & FONNUM, 1978; FONNUM *et al*., 1979). Selective changes in aspartate glutamate levels have, however, been found in both the neostriatum and thalamic nuclei as well as in the lateral septum and the nucleus accumbens after denervations (KIM, HASSLER, HAUG & PAIK, 1977; LUND-KARLSEN & FONNUM, 1978; FONNUM & WALAAS, 1978; WALAAS & FONNUM, 1979*a*; FONNUM *et al*., 1980).

In some studies (WALAAS & FONNUM, 1979*a*), focused microwave irradiation was used to achieve rapid termination of possible post-mortem changes in the endogenous amino acid concentrations. However, in preliminary studies with samples from the bed nucleus of the stria terminalis, this fixation method was found unsuitable for the present study. Instead, it was decided to use rapid decapitation followed by freezing with a CO_2-jet for the amino acid analysis. This method, which allows preparation of freeze-dried

TABLE 2. CONTENT OF SEVERAL AMINO ACIDS IN FORNIX-INNERVATED RAT BRAIN NUCLEI

	Aspartate	Glutamate	Glutamine	Taurine	Serine	GABA
Anterior diagonal band nucleus	17.8 ± 1.1 (11.5)	47.6 ± 3.2 (11.5)	28.4 ± 1.9 (11.5)	26.7 ± 2.2 (11.4)	2.8 ± 0.2 (4.2)	42.3 ± 2.0 (11.4)
Nucleus accumbens	14.8 ± 0.8 (9.3)	66.4 ± 2.9 (9.3)	43.9 ± 1.7 (9.3)	24.9 ± 2.1 (9.3)	5.2 ± 0.8 (8.3)	20.4 ± 1.7 (9.3)
Lateral septum	14.7 ± 0.8 (23.6)	60.8 ± 3.4 (23.6)	40.4 ± 2.4 (23.6)	33.2 ± 5.4 (23.6)	7.7 ± 0.9 (20.5)	27.9 ± 5.2 (23.6)
Bed nucleus of the stria terminalis	15.3 ± 1.8 (16.5)	55.4 ± 2.5 (16.5)	33.3 ± 3.2 (16.5)	15.8 ± 1.8 (15.5)	3.7 ± 0.7 (8.3)	21.3 ± 2.2 (8.3)
Mediobasal hypothalamus	18.6 ± 1.4 (13.4)	57.8 ± 3.6 (13.4)	31.1 ± 3.0 (13.4)	21.9 ± 2.2 (13.4)	3.8 ± 0.6 (13.4)	26.2 ± 2.1 (13.4)
Mammillary body	18.9 ± 1.7 (22.5)	49.5 ± 2.0 (22.5)	38.6 ± 2.9 (22.5)	13.2 ± 1.0 (21.5)	4.0 ± 1.0 (22.5)	18.2 ± 1.9 (22.5)

Results presented as μmoles/g dry weight, mean ± SEM (number of assays, number of animals).

TABLE 3. CONCENTRATIONS OF AMINO ACIDS FOLLOWING BILATERAL TRANSECTION OF THE FORNIX

	Aspartate	*Glutamate*	*Glutamine*	*Taurine*	Serine	GABA
Anterior diagonal band nucleus	81 ± 4*(16.5)	85 ± 2*(16.5)	112 ± 6 (15.5)	100 ± 6 (16.5)		85 ± 7 (12.4)
Nucleus accumbens	96 ± 6 (19.4)	66 ± 4**(19.4)	99 ± 5 (18.4)	124 ± 9 (17.4)	90 ± 5 (17.4)	125 ± 11 (15.4)
Lateral septum	85 ± 4 (35.6)	60 ± 3**(36.6)	117 ± 8 (38.6)	118 ± 9 (35.6)	114 ± 17 (33.6)	110 ± 12 (38.6)
Bed nucleus of the stria terminals	96 ± 8 (29.9)	73 ± 3**(29.9)	149 ± 18** (19.9)	138 ± 13* (29.9)	125 ± 12 (21.6)	104 ± 8 (15.6)
Mediobasal hypothalamus	86 ± 6 (17.4)	76 ± 5**(18.4)	122 ± 11 (18.4)	91 ± 11 (18.4)	100 ± 9 (18.4)	103 ± 9 (17.4)
Mammillary body	79 ± 6*(29.9)	70 ± 4**(30.9)	99 ± 7 (30.9)	106 ± 5 (30.9)	99 ± 9 (27.9)	86 ± 5 (25.8)

Results presented as per cent of unoperated controls (Table 2), mean ± SEM (number of assays, number of animals). **$P < 0.01$, *$P < 0.05$.

sections, and thus facilitates the precise dissection of the samples (Fonnum *et al.*, 1970; 1977), is not expected to influence greatly the concentration of glutamate, since the concentration of this amino acid appears to be relatively stable in the rat brain post mortem (Balcom, Lenox & Meyerhoff, 1976). However, it is not possible to ascertain the GABA concentration reliably with this method, due to the rapid post-mortem increase in the level of this amino acid (Balcom, Lenox & Meyerhoff, 1975; Van Der Heyden, De Kloet, Korf & Versteeg, 1979). The GABA levels reported in the present work are therefore probably higher than the amount of GABA present in the tissue *in vivo* (Balcom *et al.*, 1975; Fonnum & Walaas, 1978; Walaas & Fonnum, 1978; 1979*a*). However, as all brains were treated similarly, comparison between the GABA concentrations in corresponding regions from unoperated and lesioned animals is probably permissible. The lack of significant changes in GABA level agrees with the lack of effect on the GABA uptake.

Identity of the transmitter in the fornix fibres

The nuclei analysed in the present study all seem to receive excitatory allocortical fibres through the fornix and or fornix superior, as judged from anatomical and physiological studies (Raisman, Cowan & Powell, 1966; Swanson & Cowan, 1977; Meibach & Siegel, 1977; Andersen *et al.*, 1973; McLennan & Miller, 1974; Hubbard *et al.*, 1979). Furthermore, the nuclei display a very high concentration of glutamate, whereas aspartate did not correspond to more than 22–35% of the glutamate concentration. It is therefore important that the only consistent and significant neurochemical change in all these nuclei after axotomy of the fornix fibres was a considerable decrease in both the high affinity uptake of glutamate and in the tissue concentration of glutamate. In the diagonal band nucleus and in the mammillary body a small decrease in the level of aspartate was also seen. The other amino acids, including GABA, and the GABA uptake activity did not decrease significantly after this lesion in any region.

Thus, glutamate is the only amino acid transmitter candidate consistently associated with these corticofugal fibres. We therefore suggest that the majority of these allocortical efferents use this amino acid as transmitter. Minor contingents of aspartate fibres might also terminate in the diagonal band or the mammillary body. However, the very small absolute decrease in aspartate levels after lesions compared to the decrease in glutamate levels indicates that the number of putative aspartate fibres probably is very small. Alternatively, the decrease in aspartate levels might be an unspecific effect following the glutamate denervation.

Possible functional implications

These conclusions extend previous studies on the input to the septum, the nucleus accumbens and the mammillary body (Fonnum & Walaas, 1978; Storm-Mathisen & Opsahl, 1978; Nitsch *et al.*, 1979; Walaas & Fonnum, 1979*a*; Zaczek *et al.*, 1979), and some aspects of these glutamate projections have been discussed previously (Walaas, 1980*a*,*b*; Malthe-Sørenssen, Odden & Walaas, 1980). In contrast, the identification of glutamate fibres to the anterior diagonal band nucleus, the bed nucleus of the stria terminalis and the mediobasal hypothalamus deserves some comment.

The nucleus of the diagonal band. This contains a dense collection of acetylcholinesterase-positive neurons (Jacobowitz & Palkowitz, 1974), which project to the frontal cortex, septum and hippocampus (Divac, 1975; Malthe-Sørenssen *et al.*, 1980). The present study indicates that this nucleus might receive glutamate fibres from the hippocampal formation. However, these fibres apparently terminate only in the rostral part of the diagonal band nucleus, as the glutamate uptake in samples from more caudal parts of the nucleus was unchanged after fornix lesion (results not shown). Thus these results are in agreement with electrophysiological investigations in the cat brain, which have shown that excitatory fornix fibres terminate in the anterior but not the posterior diagonal band nucleus (Hubbard *et al.*, 1979). The identity of the diagonal band neurons receiving this glutamate input is uncertain. However, destruction of hippocampal and subicular neurons changes the acetylcholine level and choline uptake, but not the activity of choline acetyltransferase in the hippocampus (Schwarcz, Zaczek & Coyle, 1978; Zaczek *et al.*, 1979). This may be explained by an excitatory input from the hippocampus regulating the activity of cholinergic cell bodies projecting to the hippocampus. These cells originate mainly in the nucleus of the diagonal band (Shute & Lewis, 1967; Malthe-Sørenssen *et al.*, 1980). It is therefore tempting to suggest that some of the glutamate fibres reported in the present study may be responsible for regulation of the cholinergic cell bodies.

The bed nucleus of the stria terminalis. The results on this nucleus expand the already considerable number of transmitter candidates present in this nucleus. Previous work has demonstrated high concentrations of GABA and high activity of glutamate decarboxylase in this nucleus (Ben-Ari, Kanazawa & Zigmond, 1976; Walaas & Fonnum, 1979*b*), and some of these GABA-containing fibres appear to arrive through the stria terminalis (LeGal LaSalle, Paxinos, Emson & Ben-Ari, 1978). Due to the proximity of the fimbria fornix and the stria terminalis, we found it difficult selectively to lesion one of these bundles without affecting the other (Walaas & Fonnum, 1979*a*). However, no evidence for a destroyed GABA input, i.e. a lesioned stria terminalis, was found in any of the animals used in the present study, as measured by GABA content and GABA uptake. In contrast, the glutamate content and uptake decreased significantly in all animals. Most of the glutamate

fibres reaching the bed nucleus therefore appear to travel in the fornix. However, it cannot be excluded that some glutamate fibres also could arrive through the stria terminalis.

The hypothalamus. Most of the allocortical fibres from the hippocampal formation to the hypothalamus, which travel in the medial corticohypothalamic tracts, pass rostrocaudally through the hypothalamus between the arcuate and ventromedial nuclei (SWANSON & COWAN, 1977; MEIBACH & SIEGAL, 1977). Neurons in this part of the mediobasal hypothalamus are sensitive to glutamate-application (GELLER, 1976) and newly synthesized glutamate is released from this region *in vivo* (MEEKER & MYERS, 1979). The neurons in the arcuate nucleus are very sensitive to the neurotoxic effects of the parenteral administration of glutamate (OLNEY, 1979; WALAAS & FONNUM, 1978), and destruction of these neurons leads to gross endocrinological abnormalities (OLNEY, 1979; REDDING, SCHALLY, ARIMURA & WAKOBOYASHI, 1971; NAGASAWA, YANAI & KIKUYAMA, 1974). Also, application of non-toxic doses of glutamate or glutamate-analogs to the mediobasal hypothalamus leads to rapid changes in the release pattern of the pituitary gland (OLNEY, 1979). Thus, glutamate-neurotransmission may be important in hypothalamic function. The present results demonstrating selective decreases in both the glutamate uptake and content after transection of the fornix fibres to the hypothalamus indicate that these fibres provide a major part of the glutamate innervation in the mediobasal hypothalamus. They may thus be important in the expression of endocrine functions initiated in the hippocampal formation (AZMITIA & CONRAD, 1976; KNIGGE & HAYES, 1963; VELASCO & TALEISNIK, 1969).

Conclusions

The present study indicates that the fornix bundle in the rat brain distributes massive projections of putative glutamate-containing fibres to the septum, the basal telencephalon and the basal hypothalamus. A few aspartate-containing fibres may also enter the anterior diagonal band nucleus and the mammillary body. These results thus expand the already considerable list of corticofugal fibres which probably use an acidic amino acid as neurotransmitter (FONNUM *et al.*, 1979; WALAAS, 1980*b*).

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THE EFFECT OF PARENTERAL GLUTAMATE TREATMENT ON THE LOCALIZATION OF NEUROTRANSMITTERS IN THE MEDIOBASAL HYPOTHALAMUS

IVAR WALAAS and FRODE FONNUM

Norwegian Defence Research Establishment, Division for Toxicology, N-2007 Kjeller (Norway)

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SUMMARY

The localization of cholinergic, aminergic and amino acid-ergic neurones in the mediobasal hypothalamus has been studied in normal rat brain and in brains where neurones in nucleus arcuatus were destroyed by repeated administration of 2 mg/g body weight monosodium glutamate to newborn animals. In normal animals acetylcholinesterase staining, choline acetyltransferase and aromatic L-amino acid decarboxylase were concentrated in the median eminence and the arcuate nucleus. Glutamate decarboxylase was concentrated at the boundary between the ventromedial and the arcuate nuclei, with lower activity in the arcuate nucleus and very low activity in the median eminence. Nucleus arcuatus contained an intermediate level of high affinity glutamate uptake.

In the lesioned animals, there were significant decreases in choline acetyltransferase, acetylcholinesterase staining and glutamate decarboxylase in the median eminence, whereas choline acetyltransferase activity and acetylcholinesterase staining, but not glutamate decarboxylase activity, were decreased in nucleus arcuatus. Aromatic L-amino acid decarboxylase was unchanged in all regions studied. The high affinity uptakes of glutamate, dopamine and noradrenaline, and the endogenous amino acid levels were also unchanged in the treated animals.

The results indicate the existence of acetylcholine- and GABA-containing elements in the tuberoinfundibular tract. They further indicate that the dopamine cells in the arcuate nucleus are less sensitive to the toxic effect of glutamate than other cell types, possibly because they contain less glutamate receptors.

INTRODUCTION

The systemic administration of high doses of monosodium glutamate to newborn animals leads to neuronal necrosis in several brain regions, most consistently

in the nucleus arcuatus of the mediobasal hypothalamus[5,26,35]. In this nucleus, which through its connections to the median eminence[17] constitutes an important neuroendocrine center, 80–90% of the local neurones are destroyed after administration of 2 mg/g body weight of glutamate (GLU)[37]. In contrast, glial cells and axons passing through the nucleus are left intact, and the neighbouring ventromedial nucleus is completely unaffected[44]. The GLU-induced lesion is thus of remarkable selectivity, and seems well suited for studies of the anatomical and neurochemical organization of connections in mediobasal hypothalamus.

The aim of the present investigation was to utilize the toxic effect of GLU as a tool for studying the localization of putative neurotransmitters in mediobasal hypothalamus. Choline acetyltransferase (ChAT, E.C. 2.3.1.6), glutamate decarboxylase (GAD, E.C. 4.1.1.15) and aromatic L-amino acid decarboxylase (AAD, E.C. 4.1.1.26) were studied in microdissected freeze-dried sections from rat hypothalamus. Acetylcholinesterase (AChE, E.C. 3.1.1.7) was examined by histochemical staining. The relative abundance of noradrenaline (NA) and dopamine (DA) fibres in the arcuate nucleus and median eminence was estimated by measuring the high affinity uptake of these amines. Finally, the localization of putative amino acid transmitters was investigated by measuring the high affinity uptake of GLU and by amino acid analysis. Part of this work has been presented in a preliminary form[30].

MATERIALS AND METHODS

[1-^{14}C]acetyl-CoA, dansyl-(dimethyl-[^{3}H]amino)-chloride, L-[^{3}H]glutamic acid, L-[7,8-^{3}H]noradrenaline and 3,4-[ethyl-2-^{3}H]dopamine were obtained from New England Nuclear, Boston. L-[^{14}C]glutamic acid, DL-3,4-dihydroxy-[2-^{14}C]phenylalanine and ^{14}C-labelled amino acids were from the Radiochemical Centre, Amersham. Sodium L-glutamic acid and unlabelled dansylated amino acids were from Sigma Chemicals, St. Louis. Polyamide plates (F 1700) were from Schleicher and Schüll, D-2433 Dassel. Nialamide hydrochloride was a gift from Pfizer, benztropine was a gift from Merck, Sharp and Dohme, desmethyl-imipramine was a gift from Ciba-Geigy, and ethopropazine was from Pharma Rhodia. Methylisocyclopentylfluorophosphate (soman) was synthesized in our laboratory. White Wistar rats were obtained from Dyrlaege Mollergaard-Hanssens Avlslaboratorium, Denmark, and allowed to breed in our animals quarters.

Tissue preparation

Monosodium glutamate was dissolved in distilled water and the solution neutralized to pH 7.2. Newborn rats of both sexes were given daily subcutaneous injections of 2 mg/g body weight during their first week of life. The animals were fed normal lab chow and water ad lib, and housed together with uninjected littermates, which were used as controls. The animals were sacrificed at 6–9 weeks of age. For enzyme studies, the animals were decapitated, the brain rapidly but carefully dissected out, placed on a microtome chuck and frozen with a CO_2 jet. Serial transverse sections (40 μm) from hypothalamus were cut in a cryostat at −18°C, freeze-dried and stored

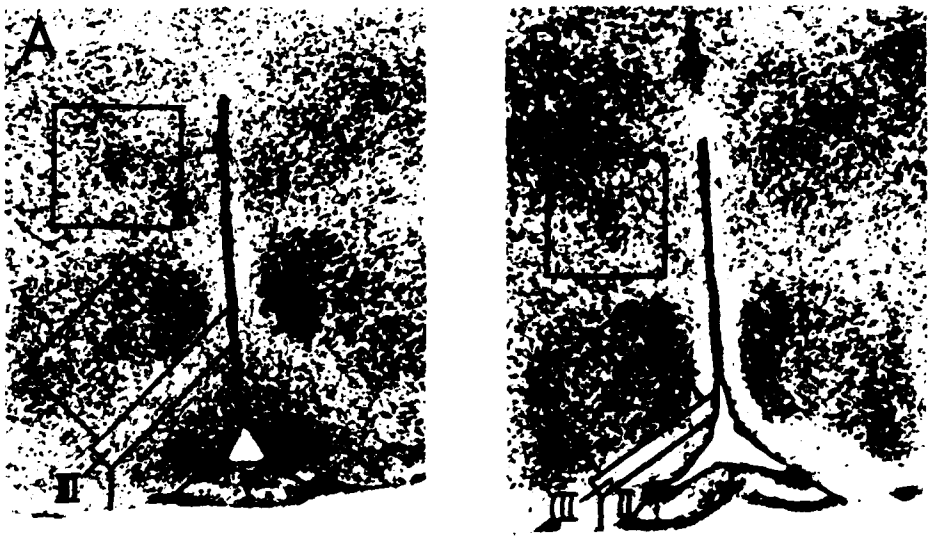

Fig. 1. A: cell-stained 40 μm section from mediobasal hypothalamus of normal rat. Dissection of freeze-dried samples is indicated on the left part of figure. I, median eminence; II, nucleus arcuatus; III, 'lateral arcuate'; IV, nucleus ventromedialis; V, nucleus dorsomedialis. B: section from GLU-treated rat, demonstrating heavy cell loss in the arcuate nucleus. Dissected samples as in A. 25, May Grünwald Giemsa stain.

as previously described[27]. For histological staining, 20–40 μm sections were collected on microscopic slides and allowed to dry at room temperature. For amino acid analysis, the animals were sacrificed by exposing their heads to a beam of focussed microwave radiation in a Litton 70/50 oven for 3 sec to rapidly inactivate all enzymes and avoid postmortem artifacts[16]. The heads were then rapidly cooled on ice and the brain carefully taken out. By this procedure, it proved impossible to preserve the median eminence, but microscopic inspection of transverse slices demonstrated the arcuate and ventromedial nuclei to be intact.

Dissection

The freeze-dried sections were compared to corresponding cell-stained sections under a stereomicroscope, and the different regions were identified after the atlas of König and Klippel[21]. The regions taken out for analysis included the median eminence, the nucleus arcuatus, the boundary between the arcuate and the ventromedial nuclei (named 'lateral arcuate'), the ventromedial nucleus and the dorsomedial nucleus (Fig. 1). The samples (0.1–2 μg dry weight) were weighed on a fish-pole quartz fiber balance[27] and assayed directly for enzyme activities.

For uptake studies, the brains were placed with the ventral surface up, and the median eminence was dissected with razor blades and small forceps under a stereomicroscope in the cold room according to Cuello et al.[8]. In addition to median

Fig. 2. Cell-stained sections from normal and GLU-treated animal, demonstrating the selective neuronal loss and shrinkage of the arcuate nucleus (ar). Me, median eminence. × 75, May Grünwald Giemsa stain.

eminence this sample contained most of the arcuate nucleus and the proximal stalk eminence. Samples were pooled and kept on ice until homogenization.

For amino acid analysis, the hypothalami of the cooled brains were frontally sliced with two razor blades, one at the level of the entrance of the optic tract into the brain and the other at the level of the stalk median eminence. From this slice, a triangular sample (Fig. 1) containing the arcuate nucleus and the medial part of the ventromedial nucleus was taken.

Histological preparation

The air-dried sections were stained with a routine May Grünwald/Giemsa method for cell staining, or for AChE by the thiocholine method as previously described[52]. AChE sections were incubated for 6–24 h, routinely using ethopropazine (10^{-4} *M*) as pseudocholinesterase inhibitor[51]. The staining was compared with sections from animals which had been pretreated with a lethal dose (0.2 μg/g body weight) of soman and killed 15 min after injection, thus inhibiting all cholinesterase.

Biochemical methods

ChAT was assayed by a previously described micromethod[12]. GAD was assayed by a CO_2-trapping method as described by Henke and Fonnum[17] with a final concentration of 20 m*M* GLU, using Triton X-100 to ensure penetration of substrate and to minimize non-GAD-released CO_2[31]. AAD was assayed as previously described[1], with final concentration of 0.3 m*M* L-DOPA. Protein was determined as described by Lowry et al.[28], with bovine serum albumin as standard.

For high affinity uptake, the pooled samples were homogenized in 40 μl 0.32 *M* sucrose at 800 rev/min in a glass-Teflon microhomogenizer. For catecholamine uptake, homogenate containing 30–40 μg protein was added to 0.5 ml Tris-buffered medium which contained 10^{-4} *M* ascorbic acid and 12.5 μM nialamide hydrochloride. Benztropine (10^{-6} *M*) followed by [^{3}H]NA (10^{-7} *M*), or desmethylimipramine (10^{-6} *M*) followed by [^{3}H]DA (10^{-7} *M*) were added, the uptake performed at 37 °C for 5 min, and terminated by Millipore filtration as previously described[13]. Benztropine and desmethylimipramine inhibited DA or NA uptake, respectively[8]. The GLU uptake was performed with 10^{-7} *M* GLU at 25 °C for 3 min in the same manner.

The amino acids were analysed by the dansylchloride method with ^{14}C-labelled amino acids as internal standards, as described in detail by Lund-Karlsen and Fonnum[29]. The dansylated amino acids were dissolved in acetone-acetic acid (3:2) before scintillation counting. Comparison between biochemical results was performed with the Wilcoxon two-sample test.

RESULTS

Previous histological examinations of the arcuate nucleus after GLU administration have shown that the lesion is characterized by neurones undergoing necrosis and phagocytosis, followed by gliosis within the first 48 h[5,26,35]. Our cell-stained sections from 7-week-old animals seem to indicate that most neuronal perikarya have disappeared irreversibly from the arcuate nucleus, while the remaining cells seem to be characterized by small nuclei and lack of Nissl substance. They thus probably represent glial cells (Fig. 2). The study of the cell-stained sections led us to make full use of our dissection technique which, in contrast to the 'punching' technique[42], allows complete sampling of regions with irregular outline. The distribution studies were performed on the median eminence, the triangular nucleus arcuatus, the almost perikarya-free region separating the arcuate and the ventromedial nuclei, and samples from the ventromedial and the dorsomedial nuclei (Fig. 1).

In normal animals, both ChAT and AAD showed low activities in the ventromedial nucleus, with higher levels in the arcuate samples and the median eminence (Table I). The highest activity of GAD was found in the region between the arcuate and the ventromedial nuclei and in the dorsomedial nucleus. The activity in the arcuate nucleus was intermediate, and the median eminence displayed low activity. The dorsomedial nucleus exhibited intermediate ChAT and AAD activity (Table I).

In the lesioned animals the levels of enzyme activities in the dorsomedial and ventromedial nuclei were unchanged. AAD was also found to be unchanged in the lateral arcuate, the arcuate nucleus proper and the median eminence. GAD was unchanged in the lateral arcuate and the arcuate nucleus, but a 50% decrease in activity was found in the median eminence. ChAT was significantly decreased in all 3 areas, but most pronounced in the arcuate nucleus and the median eminence (Table I).

AChE staining of sections from normal animals demonstrated that the ventromedial nucleus was almost devoid of staining product. The arcuate nucleus, however, did exhibit intermediate staining, in agreement with the distribution of ChAT. The

TABLE I

The effect of GLU administration on AAD, ChAT and GAD in mediobasal hypothalamus of rat

Values presented as mean ± S.E. (number of samples, number of animals).

	Normal brain (μmole/h/g dry wt)			*GLU-treated brain (per cent of normal)*		
	AAD	*ChAT*	*GAD*	*AAD*	*ChAT*	*GAD*
Median eminence	19.5 ± 2.1 (29,7)	40 ± 11 (6,4)	109 ± 13 (21,4)	93 ± 7 (32,5)	32 ± 8 (10,4)*	51 ± 3 (33,4)*
Arcuate nucleus	14.0 ± 1.4 (28,5)	24 ± 3 (19,5)	249 ± 9 (37,7)	94 ± 11 (25,5)	31 ± 5 (24,5)*	118 ± 8 (49,7)
'Lateral arcuate'	8.9 ± 1.5 (14,4)	11 ± 2 (14,4)	506 ± 56 (14,4)	90 ± 5 (12,4)	50 ± 9 (14,4)**	90 ± 10 (15,4)
Ventromedial nucleus	7.1 ± 1.1 (17,5)	4 ± 1 (12,4)	396 ± 23 (19,4)	77 ± 11 (18,5)	125 ± 20 (18,3)	96 ± 8 (10,4)
Dorsomedial nucleus	11.9 ± 1.2 (20,4)	18 ± 1 (19,4)	453 ± 24 (14,4)	86 ± 10 (17,4)	122 ± 17 (22,4)	100 ± 8 (14,4)

* Different from normal, $P < 0.001$.
** Different from normal, $P < 0.05$.

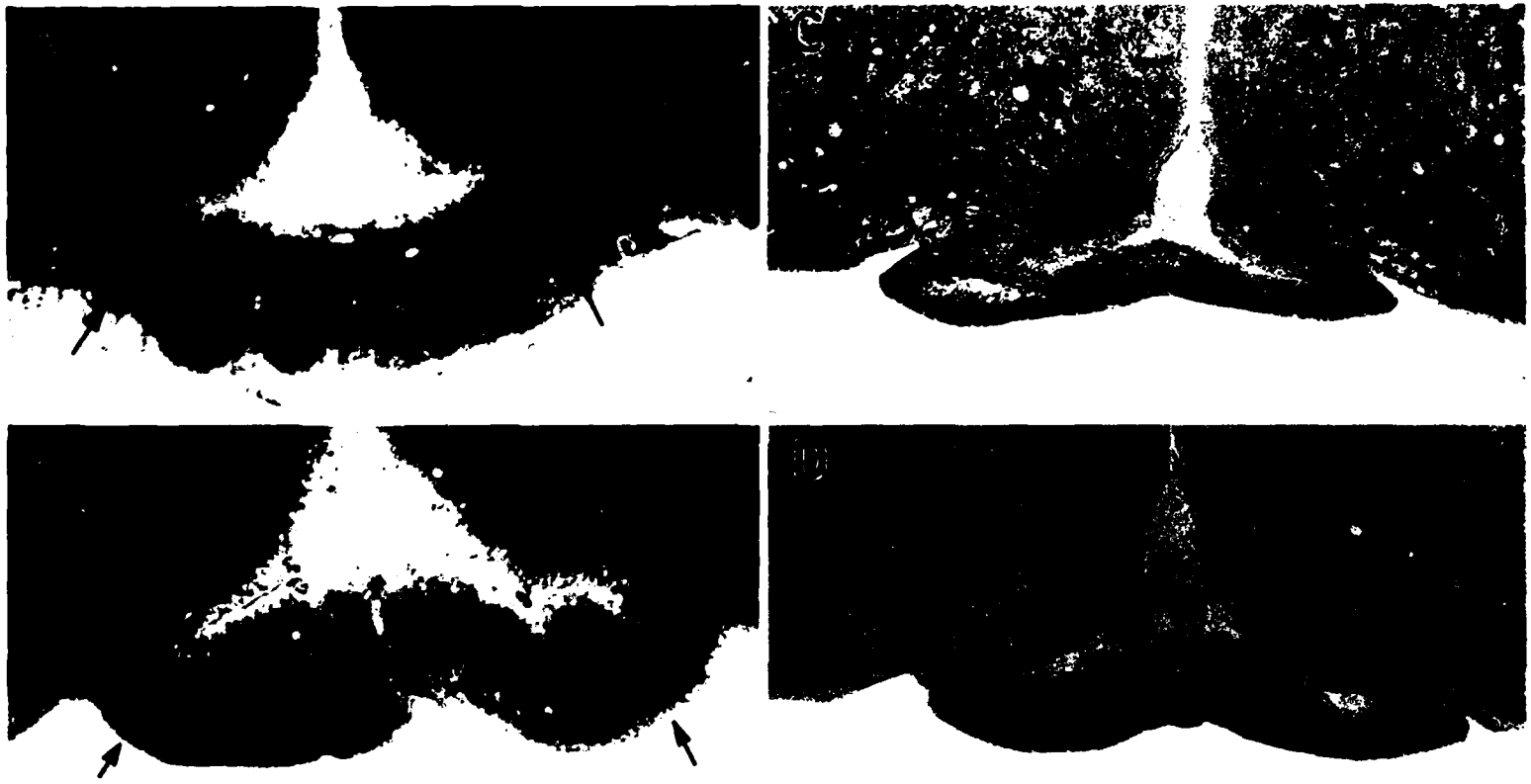

Fig. 3. 40 μm sections stained for AChE. A: rostral median eminence incubated 24 h. Staining seen in the external lateral regions (arrow). B: caudal median eminence incubated 24 h Staining seen in the external region (arrow). C: section stained for 6 h, demonstrating AChE-positive cell bodies in the arcuate nucleus (arrows). D: section stained for 6 h from GLU-treated animal, demonstrating loss of staining in median eminence and arcuate nucleus. × 75, AChE stain.

556

TABLE II

The effect of GLU-administration on the high affinity uptake of transmitter candidates in median eminence–nucleus arcuatus

Values presented as mean ± S.E. (number of experiments).

	Normal brain (nmole/min/g protein)	*GLU-treated brain (per cent of normal)*
GLU	26.2 ± 2.8 (6)	93 ± 10 (7)
DA	0.72 ± 0.09 (8)	113 ± 24 (6)
NA	0.29 ± 0.02 (8)	109 ± 6 (6)

TABLE III

The effect of GLU administration on endogenous amino acids in nucleus arcuatus–ventromedialis

Values presented as mean ± S.E.

	Normal brain (n = 6) (μmole/g protein)	*GLU-treated brain (n 9) (per cent of normal)*
ASP	35 ± 3	91 ± 9
GLU	105 ± 8	103 ± 8
GLY	26 ± 2	86 ± 13
ALA	12 ± 1	116 ± 17
GABA	41 ± 3	87 ± 6
GLN	87 ± 7	80 ± 6
TAU	57 ± 5	123 ± 15

median eminence was found to exhibit staining in the lateral, external regions, particularly in the caudal part (Fig. 3). This staining was most clearly demonstrated after long incubation time, e.g. 24 h, whereas the staining of perikarya in the arcuate nucleus was optimal after shorter incubation. After preparation of sections from animals treated with a lethal dose of soman, the staining was absent in both regions. In the GLU-injected animals the AChE staining intensity was reduced in the arcuate nucleus and median eminence, but unchanged in the other parts of the hypothalamus.

The other biochemical parameters were investigated on homogenates obtained by dissection of wet tissue samples. The high affinity uptake of GLU in the median eminence–arcuate sample was found to be of intermediate activity, being approximately 30% of that in neostriatum, which is one of the regions with the highest GLU uptake, but twice as high as the uptake in more lateral samples, and 4 times as high as the uptake in globus pallidus (data not shown). The GLU uptake was found to be unchanged in samples from lesioned animals (Table II). The high affinity uptake of catecholamines demonstrated that DA at 10^{-7} *M* was the most active substrate in agreement with a previous report[8]. The uptake activities of both DA and NA were also unchanged in the median eminence–arcuate sample from lesioned animals (Table II).

Analysis of endogenous amino acids in an arcuate–ventromedial sample demon-

strated considerable concentration of GLU, aspartic acid (ASP) and γ-aminobutyric acid (GABA) (Table III). In the lesioned animals no significant changes were detected (Table III).

DISCUSSION

Several approaches have been used for the study of hypothalamic neurotransmitters, both histochemical (for review see ref. 57), and microdissection combined with microchemical assays (for review see ref. 2). In the present investigation we have taken advantage of the selective lesion of local neurones in the nucleus arcuatus after GLU administration to newborn animals to study the origin and distribution of neurotransmitters in this region. As GLU administration to newborn or adult animals has been reported to have interesting endocrinological consequences[33,38,41,45,46], such studies would presumably also throw some light on the neurochemical basis of these phenomena.

The small dimensions of the mediobasal hypothalamus necessitate very precise dissection procedures for such studies. Initially we analyzed the enzyme activities in the median eminence in samples from fresh tissue dissected as described by Cuello et al.[8]. However, in these studies (results not shown) we did not find the same relative values and the same decreases in activity as we later found using microdissection on freeze-dried sections. This is probably due to contamination of the median eminence with neighbouring structures also containing the enzymes in question, and indicates that such samples are not suitable for detailed studies on the median eminence proper (see also ref. 7). The microdissection on freeze-dried samples allowed us to avoid such contamination. In addition, it enabled us to dissect samples of widely different outlines and weights from thin sections compared to sections used in punching*. The uptake studies, however, had to be performed on a combined median eminence arcuate sample, and the amino acid analysis on an arcuate ventromedial sample.

The involvement of acetylcholine in neuroendocrine hypothalamic function has been suspected on pharmacological and physiological grounds[15,20]. Our results demonstrate that the specific cholinergic marker ChAT[11] displays an intermediate activity in the dorsomedial nucleus, a low activity in the ventromedial nucleus, and high activity in the arcuate nucleus and median eminence. Compared to previously published results[3], we find a relatively higher activity in the arcuate nucleus, which is probably due to a more suitable dissection procedure as discussed above. In the lesioned animals, the arcuate nucleus and the median eminence displayed a 70% decrease in ChAT activity, while the activity in the other regions was unchanged. As the rat median eminence is almost devoid of nerve cells[23], this indicates the existence of a cholinergic fiber tract originating in the arcuate nucleus and terminating in the median eminence, in agreement with the independent investigation of Carson et al.[6].

* The weight of our freeze-dried median eminence samples indicates a total wet weight of 70–90 μg of the median eminence, in agreement with Chiocchio et al. .

The remaining ChAT activity in the arcuate nucleus could represent minor projections from other areas, as suggested from knife lesions by Brownstein et al.[4].

Our results with AChE staining would also support the concept of a cholinergic tuberoinfundibular tract. In sections from normal animals we found staining in the external lateral part of the median eminence, i.e. the same region which displays most intense staining for DA and for hypothalamic releasing factors[18]. Both the median eminence and nucleus arcuatus displayed considerably less intense staining in the lesioned animals, indicating that this staining represents a visualization of tuberoinfundibular fibres. In contrast, other workers[19,43] have failed to find AChE staining in the median eminence. Our conditions, by using thick sections from frozen tissue, avoiding prefixation, and using long incubation periods, would seem to favour staining of AChE. The conditions which lead to staining of median eminence are, however, at the expence of high resolution. From the effect of the cholinesterase inhibitors ethopropazine and soman we conclude that the staining observed does indicate the presence of 'true' AChE in the median eminence of the rat.

The distribution of the GABA-marker GAD[11] reported here is an extension of the results recently reported by others[55]. We here demonstrate that the highest activity of GAD in the mediobasal hypothalamus is located not in any of the nuclei, but in the region between the arcuate nucleus and the medial part of the ventromedial nucleus, with less activity in the arcuate nucleus proper. The median eminence displays much lower activity. Previous investigators have proposed that GABA may not be important in median eminence function[22,55]. However, our results demonstrate that in the lesioned animals a decrease of 50% in GAD activity occurred in the median eminence, while the rest of the hypothalamus had unchanged activities. This could be due either to destruction of a GABAergic fiber tract terminating in the median eminence or the destruction of intrinsic GABAergic neurones in the median eminence. However, such intrinsic cells are reported to be very scarce[23], and we therefore think it is most likely that our results indicate the existence of a GABAergic tuberoinfundibular tract, which originates in the arcuate nucleus. This tract could possibly represent the anatomical basis for the endocrinological effects of GABA[20,39,40]. The small and insignificant changes in GAD activity found in the arcuate nuclei of the lesioned animals probably mean that most of the GAD activity in this nucleus is concentrated in afferent terminals, and only a small proportion is located in local nerve cells. This is in agreement with the results of other studies, which demonstrate that a major part of GAD disappears in the arcuate nucleus but not in the median eminence after deafferentiation of the mediobasal hypothalamus[4].

The well-known DA-containing tuberoinfundibular tract, originating in the A_{12} cell group in the arcuate nucleus[56], was also studied. AAD is found in both DA-, NA- and indoleamine-containing cells, in addition to being localized in capillaries and possibly glial cells[21,25]. However, the relative values of AAD reported in this study are in good agreement with the corresponding values of tyrosine hydroxylase[48,49], which in these regions is acknowledged as being a selective marker for DA elements[1]. We also analyzed the high affinity uptake of DA and NA in median eminence–arcuate samples in order to detect any loss of DA structures[14]. However, our results indicate

that the DA innervation of the arcuate nucleus and median eminence has not been significantly destroyed in the lesioned animals. Amine fluorescence in the arcuate nucleus has been shown to disappear after acute injection of the GLU analogue kainic acid[36] to adult animals[32] or after repeated injections of very high doses (4 mg/g body wt) of GLU to newborn animals[31]. Our results indicate that these DA cells, despite this apparent GLU sensitivity, are more resistant to the neurotoxic effect of GLU than other types of cells in the arcuate nucleus. This is in agreement with results from other regions in brain and in retina[29,50].

Except for GABA, the amino acid transmitter candidates have received relatively scant attention in the hypothalamus. The preferential release of GABA, GLU and ASP from hypothalamic synaptosomes has been demonstrated [10], and it has also been shown that both systemic and intraventricular application of GLU has endocrine effects[38,41]. This endocrine action of GLU is probably located in the arcuate nucleus, as this is the only hypothalamic region where GLU is accumulated following systemic administration[44]. Our results on the high affinity uptake of GLU seem to indicate that this amino acid could be a transmitter in the median eminence–arcuate complex[9,53]. The results do however indicate that the 'glutamatergic' terminals have survived the accumulation of GLU in the arcuate nucleus, and argue against a major population of intrinsic glutamatergic cell bodies in this region. Alternatively, the glutamatergic cell bodies could be resistant to GLU accumulation. The levels of endogenous amino acids in the arcuate nucleus and medial part of the ventromedial nucleus did not show any decrease in the lesioned animals, also arguing against a major population of 'amino acid-ergic' cells being destroyed by the GLU lesion.

In conclusion, this study extends and confirms previous findings on the localization of neurotransmitters in mediobasal hypothalamus based on knife lesions and punching technique[3,4,48,54,55]. We suggest that the tuberoinfundibular tract, in addition to the previously described peptidergic and aminergic elements, contains cholinergic and GABAergic fibres. These are destroyed by GLU administration, and therefore probably originate in nucleus arcuatus. This destruction could be a part of the neurochemical basis for the endocrine dysfunction in the GLU-treated animals. Also, we find that the DA cells in this region are resistant to 2 mg/g body weight of GLU. They therefore probably do not contain a high number of GLU receptors. Finally, a relatively high concentration of 'glutamatergic' terminals was found in the arcuate region, which is probably not due to intrinsic neurones.

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560

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The Effects of Kainic Acid Injections on Guanylate Cyclase Activity in the Rat Caudatoputamen, Nucleus Accumbens and Septum

Ivar Walaas

Norwegian Defence Research Establishment, Division for Toxicology, Kjeller N-2007, Norway

Abstract: The activity of soluble and particulate guanylate cyclase (EC 4.6.1.2) has been compared with the distribution of neurotransmitter candidates in three rat forebrain nuclei, and the effects of local kainic acid injections into these nuclei have been tested. Soluble guanylate cyclase was highly concentrated in both the caudatoputamen and the nucleus accumbens, with lower activity found in the septum. This distribution coincided with markers for acetylcholine and monoamines, but not with markers for γ-aminobutyrate (GABA) or glutamate neurons. In contrast, particulate guanylate cyclase was equally active in all regions. Local injections of kainic acid, which destroyed cholinergic and GABA neurons in the caudatoputamen and in the nucleus accumbens, caused a rapid (70–90%) decrease in the soluble guanylate cyclase and a slower 50–60% fall in the particulate guanylate cyclase in these nuclei. In the septum, where kainate destroyed GABA cells but not cholinergic neurons, the guanylate cyclase activity was unchanged after the lesion. Thus, both the soluble and particulate guanylate cyclases appear to be concentrated in local neurons in the caudatoputamen and nucleus accumbens. In the septum, however, most of the guanylate cyclase activity is located outside kainate-sensitive neurons. **Key Words:** Cyclic GMP—Neurons—Glia—Forebrain nuclei. Walaas I. The effects of kainic acid injections on guanylate cyclase activity in the rat caudatoputamen, nucleus accumbens and septum. *J. Neurochem.* 36, 233–241 (1981).

Guanylate cyclase (EC 4.6.1.2), the enzyme synthesizing guanosine 3′,5′-monophosphate (cyclic GMP) (Hardman and Sutherland, 1969), is present in brain in both soluble and particulate forms (Nakazawa and Sano, 1974; Kimura and Murad, 1975; Deguchi et al., 1976; Troyer and Ferrendelli, 1976; Troyer et al., 1978). The physiological functions these enzymes possibly have in the brain are largely unknown (Ferrendelli, 1978; Greengard, 1978), but a 'second messenger' role has been suggested for cyclic GMP in mediating neurotransmission due to acetylcholine (ACh) (Lee et al., 1972; Stone and Taylor, 1977; Hanley and Iversen, 1978; Woody et al., 1978) and glutamate (Mao et al., 1974; Biggio et al., 1978). It was therefore deemed of interest to compare in detail the topographical and cellular distribution of the soluble and particulate guanylate cyclases in the caudatoputamen, nucleus accumbens and the septum, brain regions with relatively well-defined glutamate and ACh neuronal systems (Fonnum and Walaas, 1978; 1979; Walaas and Fonnum, 1979). For this purpose, the kainic acid lesioning technique has been used to distinguish between local nerve cells and glial cells (Hattori and McGeer, 1977; Schwarcz and Coyle, 1977; Coyle et al., 1978; Fonnum and Walaas, 1978; Malthe-Sørenssen et al., 1980), and biochemical markers for putative ACh, γ-aminobutyrate (GABA), monoamine and glutamate nerve terminals and glial membranes have been analysed to-

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Address correspondence and reprint requests to Ivar Walaas, Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510, U.S.A.

Abbreviations used: AAD, Aromatic L-amino acid decarboxylase; ACh, Acetylcholine; ChAT, Choline acetyltransferase; GABA, γ-Aminobutyric acid; GAD, L-Glutamate decarboxylase.

gether with the soluble and particulate guanylate cyclase activities.

Initial results from the caudatoputamen indicated a decrease in guanylate cyclase after kainate lesions, and thus a neuronal localization of the enzyme. However, a simultaneous report presented evidence suggesting that kainate-induced neuronal necrosis increased the guanylate cyclase activity in rat caudatoputamen homogenates (Hjornhammar et al., 1979). The present study has therefore also investigated some characteristics of the enzyme and confirmed that the radiochemical product formed in the present assay method is cyclic GMP.

Some preliminary results have been presented in abstract form (Walaas, 1979).

MATERIALS AND METHODS

Male Wistar rats (180–200 g body weight) were obtained from Møllegaard, Denmark. [α-^{32}P]GTP was obtained from New England Nuclear, Boston, Massachusetts, and The Radiochemical Centre, Amersham, U.K. [8-^{3}H]cyclic GMP, [8-^{3}H]5′ GMP and L-[U-^{14}C]glutamic acid were from The Radiochemical Centre, Amersham, U.K. [8-^{3}H]cyclic GMP, [8-^{3}H]GTP, [8-^{3}H]5′ AMP, L-[2,3-^{3}H]glutamic acid, DL-[2-^{14}C]dihydroxyphenylalanine and [1-^{14}C]acetyl CoA were from New England Nuclear. Guanine and adenine nucleotides (sodium salts), 3-isobutyl-1-methylxanthine, kainic acid (lot 117C-0044) and 3′,5′-cyclic nucleotide phosphodiesterase (EC 3.1.4.17) from beef heart were from Sigma Chemical Co., St. Louis, Missouri. Dowex 50W × 4 (H$^+$ form, 200–400 mesh) and Dowex 1 × 2 (Cl$^-$ form, 200–400 mesh) were from Fluka AG, Buchs, neutral alumina (grade I) was from Woelm, Eschwege, and polyethylenimine) cellulose F DC Fertigplatten (Art 5725) were from Merck, Darmstadt, West Germany. Other chemicals were commercially obtained. Chromatographic columns (0.5 × 10 cm) were from Bio-Rad (California).

Tissue Preparation

The animals were anaesthetised with fentanyl citrate and fluanisone (Hypnorm vet, Mekos, 0.1 ml/s.c.) and Valium (Roche, 0.75 mg i.p.). Then, 10 nmol kainic acid dissolved in 1 μl 140 mM sodium phosphate buffer, pH 7.4, was infused into the caudatoputamen at coordinates A 9000, L 2000 and DV − 7000 (König and Klippel, 1963) for 3 min. 5 *nmol kainate in 0.5 μl* buffer was infused into the nucleus accumbens at A 9400, L 1200 and DV − 100 as described by Walaas and Fonnum (1979), and 10 nmol kainate in 0.5 μl buffer was infused into the septum at A 7200, L 600 and DV − 1000 for 2 min (Malthe-Sørenssen et al., 1980). The needle was left in position for 5 min before withdrawal to *prevent* leakage. The animals were decapitated at the indicated survival times, and 0.6–0.8-mm frontal brain slices were prepared with a tissue chopper at 4°C. The caudatoputamen was dissected between approximate levels A 9600 and A 6500, the nucleus accumbens was taken anterior to A 8400, and the septum was taken between A 8600 and A 6900 (König and Klippel, 1963) under microscopic guidance. The septal sample contained both the medial and lateral septal nuclei, but avoided the nucleus of the diagonal band. For uptake studies, the samples were homogenised in cold 0.32 M-sucrose–5 mM-Tris-HCl, pH 7.4 (2% w/v) with 10 strokes in glass–Teflon homogenisers at 850 r.p.m., kept on ice and analysed for uptake activity within 30 min. For analysis of enzyme activities, the samples were homogenised in 20 vol. of cold 30 mM-Tris-HCl, pH 7.5, and placed on ice. The marker enzymes were analysed after addition of 0.2% Triton X-100 (w/v).

Guanylate cyclase was studied after addition of dithiothreitol (final concentration 5 mM) to the Tris-buffered homogenates. Subcellular fractionation of aliquots from the latter samples was effected by centrifugation at 4°C for 30 min at 48000 *g*, which has been found to completely separate the soluble and particulate guanylate cyclases in brain (Hoyer et al., 1978). The supernatants (i.e. soluble fractions) were collected on ice, while the pellets were washed in thiol-containing buffer, recentrifuged and suspended in their original volume of cold 30 mM-Tris-HCl–5 mM-dithiothreitol, pH 7.5, by means of glass–Teflon homogenisers. Some preparations were then extracted with 1% Triton X-100 (v/v) at 4°C for 60 min before analysis (Durham, 1976).

Biochemical Methods

Protein was analysed by the method of Lowry et al. (1951) with bovine serum albumin as standard. Blanks containing dithiothreitol were included, to correct for interference by the thiol reagent where necessary. Previously described methods were used for analysis of aromatic L-amino acid decarboxylase (AAD, EC 4.1.1.28) (*Bioch and Fonnum*, 1972), choline acetyltransferase (ChAT, EC 2.3.1.6) (Fonnum, 1975), glutamate decarboxylase (GAD, EC 4.1.1.15) (Fonnum et al., 1970), the mitochondrial enzyme carnitine acetyltransferase (EC 2.3.1.7) (Stetri and Fonnum, 1980), and the membrane enzyme 5′-nucleotidase (EC 3.1.3.5), which was analysed with 0.02 mM-[^{3}H]AMP as substrate (Avruch and Wallach, 1971). High-affinity uptake of L-[2,3-^{3}H]glutamic acid (final concentration 10^{-5} M) was studied with Millipore filtration used to terminate the incubation as described (Fonnum et al., 1977; Walaas and Fonnum, 1979). The radioactivity accumulated on the filters was determined after addition of Filtercount scintillation liquid (Packard) by liquid scintillation counting in a Packard Tricarb Model 3380 fitted with an absolute activity analyser set for ^{3}H analysis.

Guanylate cyclase was analysed by a modification of the method described by Krishna and Krishnan (1975) and Birnbaumer et al. (1979). The incubation mixture contained (final concentration): 30 mM-Tris-HCl, pH 7.5, with 0.5 mM-GTP containing 1.2×10^{6} d.p.m. [α-^{32}P]GTP and 4 mM-$MnCl_2$ as substrate, 1 mM-cyclic GMP and 1 mM-isobutylmethylxanthine to prevent breakdown of the cyclic GMP formed, approximately 10,000 c.p.m. [^{3}H]cyclic GMP to monitor recovery, and 10 μl enzyme preparation or homogenisation buffer blanks, in a total volume of 50 μl. When indicated, NaN_3 (final concentration 1 mM) or NaF (final concentration 20 mM) was added to the test tubes at the start of the preincubation. After 5 min preincubation at 37°C, the reaction was initiated by addition of GTP, $MnCl_2$ and tritiated cyclic GMP, and terminated after 5 min incubation at 37°C by addition

of 50 μl stop solution (containing 2 mM cyclic GMP, 2 mM GTP, 10 mM EDTA, and 1% sodium dodecyl sulphate). Cyclic GMP was purified by double column chromatography through Dowex 50 and alumina columns (Salomon et al., 1974; Krishna and Krishnan, 1975; Bartschmid et al., 1979). A volume of 3 ml of the alumina column eluate was added to 6 ml Dilusolve scintillation liquid (Packard) and ^{32}P and ^{3}H radioactivity was measured by double isotope liquid scintillation counting (Kobayshi and Maudsley, 1974) in a Packard Tricarb, Model 3380. Separate experiments showed that a 1 mM cyclic GMP 'trap' alone was insufficient to prevent breakdown of the radioactive cyclic GMP formed in homogenates from the caudatoputamen (data not shown). Further, it was ascertained that guanylate cyclase activity was proportional to protein content and incubation time under all conditions employed.

To identify the ^{32}P-containing product, eluates were lyophilised, dissolved in H_2O and subjected to column chromatography on Dowex 50 (Bartschmid et al., 1979) or on Dowex 1 × 2 (Cl⁻ form) (Mao and Guidotti, 1974) with or without prior incubation at 30°C for 60 min in the presence of cyclic nucleotide phosphodiesterase, 10 mM Tris HCl, pH 7.4, and 1 mM MgCl. Other samples were added to a carrier solution of cyclic GMP, GMP, GDP and GTP, and cyclic AMP, AMP, ADP and ATP, and subjected to two-dimensional ion exchange thin layer chromatography on polyethyleneimine cellulose plates (Randerath and Randerath, 1964). The nucleotide spots were visualized with UV light, transferred to counting vials, 10 ml Dilusolve was added, and ^{32}P content was analysed by liquid scintillation counting.

Statistical analysis of the results was done with the Wilcoxon two sample test (Hodges and Lehmann, 1964). Kainate lesioned septal samples were compared to septa taken from unoperated animals, while samples from the nucleus accumbens and caudatoputamen were compared to control samples from both unoperated animals and from the uninjected side of lesioned animals. Initial studies showed that the biochemical activities in the latter samples were unaffected by the kainate lesions.

RESULTS

Guanylate cyclase was first characterized in preparations from the caudatoputamen. Incubation of homogenates with [α-^{32}P]GTP followed by thin layer chromatography of the purified product showed ^{32}P radioactivity in the cyclic GMP spot only (not shown). Further, all ^{32}P in the purified product behaved like authentic cyclic GMP on both Dowex 50 and Dowex 1 columns, while more than 96% of the ^{32}P label co-migrated with ^{3}H GMP on Dowex 50 after phophodiesterase treatment (data not shown). Thus, all ^{32}P label in the purified product appeared to represent cyclic GMP. Further studies showed that guanylate cyclase activity was slightly lower in caudatoputamen homogenates prepared in dithiothreitol containing buffer compared with preparations without the thiol reagent (40.5 ± 3.3 versus 66.5 ± 7.9 pmol/min/mg protein, mean ± S.E.M., n = 8). Thiol-containing preparations avoid a possible oxidative activation of guanylate cyclase (Böhme et al., 1974; Goldberg et al., 1978), and they were therefore used in the rest of this study. The soluble enzyme thus prepared from the caudatoputamen exhibited classical Michaelis-Menten kinetics, with a K_m for GTP of 47 μM (mean of two determinations), similar to previously reported values from rodent brain (Troyer and Ferrendelli, 1976; Troyer et al., 1978). Addition of 1 mM-NaN_3 to the different preparations increased the guanylate cyclase activity in homogenates and particulate fractions 3.5- and 15-fold, respectively, but left the soluble activity almost unchanged (Table 1). Similarly, preincubation with Triton X-100 increased the homogenate and particulate activities selectively (Table 1). In contrast, incubation with 20 mM-NaI did not increase cyclic GMP synthesis in any of the fractions (data not shown). NaN_3 was also effective in activating cyclic GMP synthesis in homogenates (5-fold) and particulate fractions (11-fold) prepared from kainate lesioned caudatoputamen (data from Fig. 2). These results all conform to the properties previously described for guanylate cyclase in mammalian brain (Kimura and Murad, 1975; Kimura et al., 1975; Troyer et al., 1978).

Characterization of the neurotransmitter composition of the regions under study showed that the monoamine marker enzyme AAD and the ACh marker enzyme ChAT were concentrated in the nucleus accumbens and the caudatoputamen, while the GABA marker enzyme GAD was most active in the septum and nucleus accumbens (Table 2). The glutamate uptake capacity was greatest in the caudatoputamen. The activity of 5'-nucleotidase was concentrated in the septum and the nucleus accumbens, while the general mitochondrial marker carnitine acetyltransferase was evenly distributed (Table 2). Guanylate cyclase was most concentrated in homogenates from the caudatoputamen and nucleus accumbens, and this was apparently due to the very active soluble enzyme present in these nuclei (Table 1). In contrast, the particulate guanylate cyclase was evenly distributed in all regions, whether analysed in untreated preparations or after detergent extraction (Table 1).

The effects of kainic acid were tested in some detail in the caudatoputamen. Two days after injection of 10 nmol kainate, homogenates from this nucleus had lost 60–70% of the GAD and ChAT activities present, while guanylate cyclase was decreased by 50–60% both in untreated (Fig. 1), detergent-extracted or azide-activated homogenates (Fig. 2) when compared with similarly treated control preparations. Carnitine acetyltransferase was decreased by 30% and 5'-nucleotidase was decreased by 15%, while AAD activity was unchanged (Fig. 1). Separate analysis confirmed that most of the soluble guanylate cyclase had disappeared after this lesion (Fig. 2). In contrast, the untreated particulate preparation displayed significantly higher

TABLE 1. *Regional and subcellular distribution of untreated, detergent-extracted and NaN_3-activated guanylate cyclase in rat forebrain nuclei*

Guanylate cyclase	Caudatoputamen (n = 8)	Nucleus accumbens (n = 6)	Septum (n = 5)
Homogenate	40.5 ± 3.3	40.0 ± 3.5	17.5 ± 1.9
+ Triton X-100	149.0 ± 6.3	171.7 ± 16.7	125.5 ± 7.8
+ NaN_3	129.6 ± 17.0	-	-
Soluble	240.8 ± 12.6	224.7 ± 29.6	53.5 ± 5.1
+ Triton X-100	243.2 ± 19.3	-	-
+ NaN_3	248.1 ± 21.6	-	-
Particulate	16.3 ± 1.6	15.2 ± 0.5	14.8 ± 1.0
+ Triton X-100	123.0 ± 13.4	144.9 ± 4.7	148.0 ± 21.7
+ NaN_3	225.6 ± 9.8	-	-

Results are expressed as pmol min mg protein and are presented as mean ± S.E.M. Samples were prepared in buffer containing 5 mM dithiothreitol. Triton X-100 or NaN_3 added as described in text.

TABLE 2. *Regional distribution of AAD, ChAT, GAD, 5′-nucleotidase, carnitine acetyltransferase and high affinity glutamate uptake in rat forebrain nuclei*

	Caudatoputamen (n = 8)	Nucleus accumbens (n = 6)	Septum (n = 5)
AAD	0.93 ± 0.13	0.98 ± 0.14	0.17 ± 0.03
ChAT	3.13 ± 0.29	2.75 ± 0.13	1.10 ± 0.13
GAD	2.13 ± 0.15	3.20 ± 0.28	4.08 ± 0.11
5′-Nucleotidase	5.41 ± 0.62	8.90 ± 0.34	9.62 ± 0.31
Carnitine acetyltransferase	34.3 ± 0.3	37.5 ± 4.3	37.3 ± 2.8
Glutamate uptake	0.069 ± 0.008	0.044 ± 0.005	0.045 ± 0.004

Results are expressed as nmol min mg protein and are presented as mean ± S.E.M.

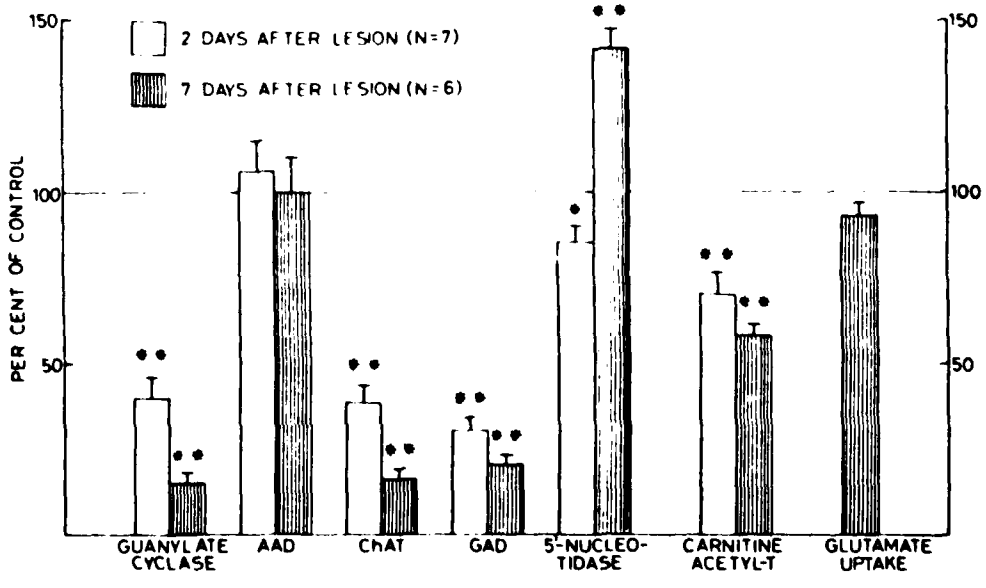

FIG. 1. Biochemical activities in rat caudatoputamen homogenates following local injection of 10 nmol kainic acid. Results presented as mean ± S.E.M., percent of control (cf. Table 1 and 2). Significantly different from control: single asterisk, $p < 0.05$, double asterisk, $p < 0.01$.

guanylate cyclase activity than similar control preparations at this time, while the NaN_3-activated particulate activity was slightly, and the detergent-extracted particulate activity was significantly, decreased compared with similarly treated control preparations (Fig. 2).

Seven days after the lesion, GAD and ChAT activities in the caudatoputamen were reduced by 80–85%, carnitine acetyltransferase was reduced by 40%, while 5'-nucleotidase now was significantly increased to about 150% of the initial activity (Fig. 1). AAD activity and glutamate uptake were unchanged. At this time point, guanylate cyclase in the different homogenate preparations was decreased by 70–80%, while the soluble activity was decreased by about 80%. The increase in the untreated particulate activity had disappeared, and a considerable decrease was found in this fraction also irrespective of treatment (Fig. 2).

The other nuclei were analysed 10 days after kainate injections, and detergent extraction was routinely used on homogenates and particulate fractions to express maximal particulate guanylate cyclase activity. Injection of 5 nmol kainate into the nucleus accumbens decreased guanylate cyclase activity in such Triton treated homogenates from this nucleus by 70%, this decrease representing an approximate 90% fall in the soluble activity and a 60% fall in the particulate activity (Fig. 3). In contrast, guanylate cyclase in the septum was unchanged after septal injection of 10 nmol kainate when tested both in homogenates (Fig. 4) and in soluble and particulate fractions (not shown). The kainate lesion of the nucleus accumbens destroyed 75–85% of the GAD and ChAT activities present, while septal injections destroyed 70% of the GAD activity but left the ChAT activity in the septum unchanged. 5'-Nucleotidase had increased considerably, while carnitine acetyltransferase was decreased in both regions (Figs. 3 and 4).

DISCUSSION

The aim of the present investigation was to identify some properties of the guanylate cyclase activities in the rat caudatoputamen, nucleus accumbens and septum. In the first part of the study, subcellular fractionation demonstrated guanylate cyclase activity in both soluble and particulate preparations from all three regions, with the soluble activity most concentrated in the caudatoputamen and nucleus accumbens. The distribution of this soluble guanylate cyclase coincided with the activity of the cholinergic marker ChAT (Fonnum et al., 1977), but not with the high-affinity uptake of glutamate, a putative marker for glutamate nerve terminals (Fonnum and Walaas, 1978; Walaas and Fonnum, 1979). Interestingly, acetylcholine has

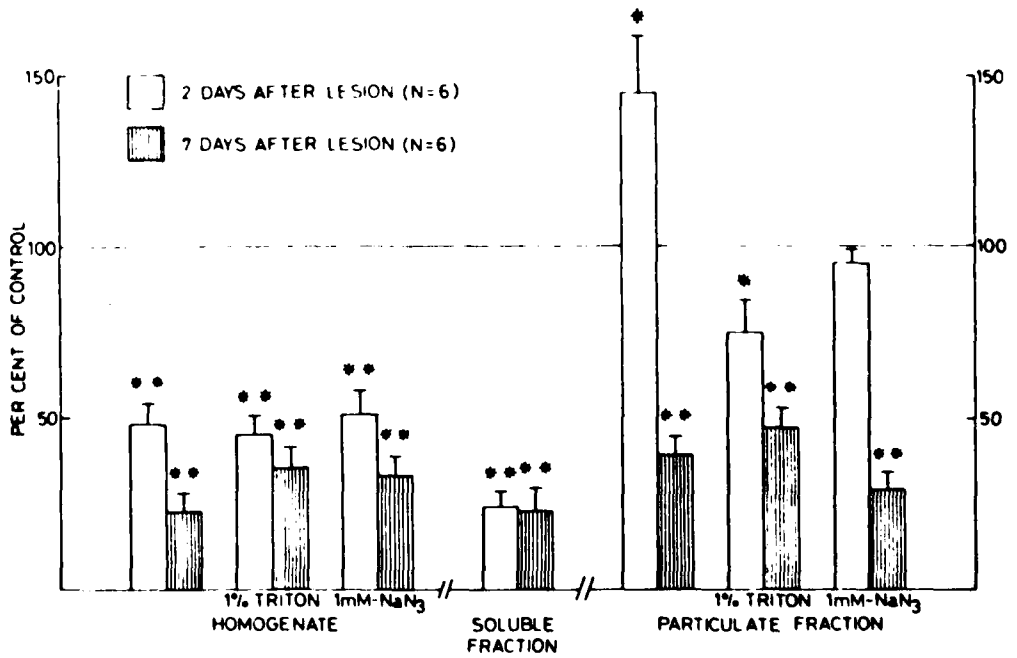

FIG. 2. Guanylate cyclase in homogenates and subcellular fractions from rat caudatoputamen after local injection of 10 nmol kainic acid. Samples prepared in 30 mM-Tris-HCl, pH 7.5 plus 5 mM-dithiothreitol. Results represent percent of similarly treated control preparations (Table 1), and are given as mean ± S.E.M. Single asterisk, $p < 0.05$, double asterisk, $p < 0.01$.

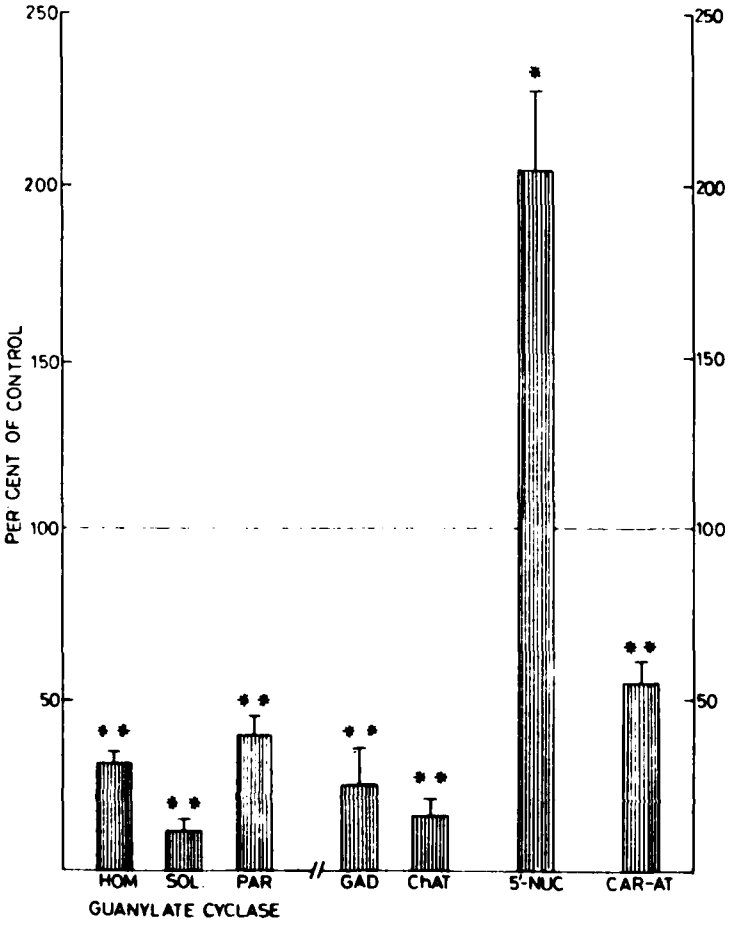

FIG. 3. Biochemial activities in rat nucleus accumbens 10 days after local injection of 5 nmol kainic acid. Guanylate cyclase was analysed in homogenates (HOM), soluble fractions (SOL) and particulate fractions (PAR), the other activities were studied in homogenates. Results presented as in Figs 1 and 2. 5'-NUC, 5'-nucleotidase. CAR-AT, carnitine acetyltransferase. Significance: single asterisk, $p < 0.05$, double asterisk, $p < 0.01$, $n = 6$.

been suggested to increase cyclic GMP levels in the caudatoputamen through an activation of the soluble guanylate cyclase only (Hanley and Iversen, 1978). In contrast, both the untreated and the more active detergent-extracted particulate guanylate cyclase activity was evenly distributed between the nuclei, unlike the neuronal markers. This difference in distribution pattern between the two guanylate cyclases is thus in general agreement with previous studies from other brain regions (Nakazawa and Sano, 1974; Greenberg et al., 1978; Troyer et al., 1978).

The cellular localization of guanylate cyclase in these regions was then studied with the kainic acid lesioning technique. Previous studies have described the local neuronal necrosis and glial cell proliferation induced by this agent in the caudatoputamen (Hattori and McGeer, 1977; Schwarcz and Coyle, 1977; Coyle et al., 1978), the nucleus accumbens (Walaas and Fonnum, 1979), and the septum (Malthe-Sørenssen et al., 1980). The present biochemical results are in agreement with these reports. In the caudatoputamen, the local ACh and GABA neurons were destroyed and the afferent monoamine and glutamate fibres were intact 1 week after kainate injection. In addition, 5'-nucleotidase, which in the brain appears to be confined to glial membranes (Kreutzberg et al., 1978), increased in activity in parallel with the impressive reactive astrocytosis that follows kainate lesions (Coyle et al., 1978). It is therefore suggested that an increase in the activity of this enzyme might reflect proliferation of glial cells. Indeed, injury-induced proliferation of 5'-nucleotidase-staining glial cells has been reported in other regions (Kreutzberg et al., 1978).

The destruction of local neurons and accompanying gliosis led in the caudatoputamen to a consistent decrease in the homogenate guanylate cyclase activity, irrespective of pretreatment, and at both 2 and 7 days after the lesion. Thus, most of the guanylate cyclase activity in the caudatoputamen appeared to be concentrated in local neurons. In agreement, both the soluble and the detergent-

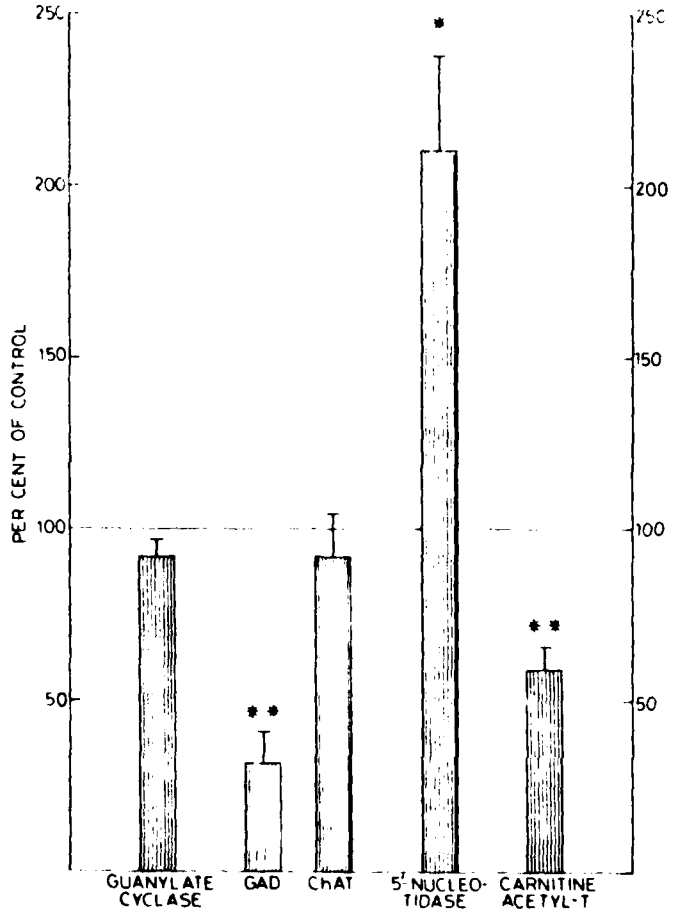

FIG. 4. Biochemical activities in homogenates from rat septum 10 days after local injection of 10 nmol kainic acid. Results presented as in Fig. 1. Single asterisk, $p < 0.05$; double asterisk, $p < 0.01$; $n = 7$.

extracted particulate activities decreased considerably after kainate lesions, on time scales similar to the rapid degeneration of neuronal cytosol and the more slowly evolving degeneration seen in the particulate nerve cell components after kainate lesions of the caudatoputamen (Hattori and McGeer, 1977; Coyle et al., 1978). The non-detergent-treated particulate guanylate cyclase presented a more complicated response, however. In most tissues, this particulate enzyme is present in a partly occluded form, and the full activity is only expressed after detergent treatment (Kimura and Murad, 1975; Durham, 1976; Goldberg and Haddox, 1977). In view of the consistent decrease in such detergent-extracted particulate activity, it was not expected that a significant increase in the non-detergent-treated particulate guanylate cyclase activity would be found 2 days after lesion. Thus, despite an apparent decrease in total particulate guanylate cyclase, the kainate lesion also appeared to induce a reversible, detergent-sensitive activation of the remaining particulate enzyme during the initial phase of degeneration. A complete characterization of this phenomenon will require further study.

In conclusion, both the soluble and particulate guanylate cyclases appear to be concentrated in local neurons in the rat caudatoputamen. A preferential neuronal localization for guanylate cyclase has also been suggested from previous studies on soluble guanylate cyclase from whole rat brain (Goridis and Morgan, 1973) and from studies using cell cultures from chick brain (Goridis et al., 1974). However, a glial localization was recently suggested, based on an increase in apparent guanylate cyclase activity in kainate-lesioned rat caudatoputamen (Tjörnhammar et al., 1979). The different guanylate cyclase methods were therefore compared during a collaboration with the latter authors. Despite using aliquots from the same homogenates, the radioimmunoassay analysis performed by Tjörnhammar et al. still showed a pronounced increase in apparent guanylate cyclase activity, while the present radiochemical method showed a 60% decrease in activity 2 days after lesion (results not

shown). The cause of this discrepancy has, then, not been identified. However, the apparent *reliability* of the present radiochemical method (Krishna and Krishnan, 1975; Craven and De Rubertis, 1976; Birnbaumer et al., 1979; this paper) suggests that radioimmunoassay of cyclic GMP formed in kainate-lesioned caudatoputamen homogenates might be subject to interfering factors.

The results from the nucleus accumbens extend our previous studies (Fonnum et al., 1977; Walaas and Fonnum, 1979) and show that the neurochemical characteristics of this nucleus are virtually identical to those of the caudatoputamen. Similar guanylate activities were found in all types of preparations, and all neurochemical markers responded similarly to kainate lesions. Thus, both guanylate cyclases are predominantly located in local neurons in the nucleus accumbens, as in the caudatoputamen. In the septum, however, kainate selectively destroyed the GABA neurons but left the guanylate cyclase activities intact. Thus, most of the cyclic GMP synthesis in this region appears to take place in cells not destroyed by kainate, such as glial cells or the ACh neurons in the medial septum (Zaczek et al., 1979; Malthe-Sørenssen et al., 1980). Kainate sensitivity may be restricted to neurons receiving glutamate fibres (Herndon and Coyle, 1977; McGeer and McGeer, 1978; Biziere and Coyle, 1978; Köhler et al., 1978; Malthe-Sørenssen et al., 1980). In the septum, such glutamate-innervated neurons, therefore, appear to contain very low guanylate cyclase activity, a conclusion which argues against a general 'second messenger' role for cyclic GMP in glutamate neurotransmission (Mao et al., 1974; Biggio et al., 1978) or kainate neurotoxicity (Honegger and Richelson, 1977).

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THE DISTRIBUTION AND ORIGIN OF GLUTAMATE DECARBOXYLASE AND CHOLINE ACETYLTRANSFERASE IN VENTRAL PALLIDUM AND OTHER BASAL FOREBRAIN REGIONS

IVAR WALAAS and FRODE FONNUM

Norwegian Defence Research Establishment, Division For Toxicology, N-2007 Kjeller (Norway)

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SUMMARY

The distribution of choline acetyltransferase (ChAT) and glutamate decarboxylase (GAD), and the histochemical reaction for acetylcholinesterase have been studied in the basal forebrain and globus pallidus of unoperated rats and in rats with an electrolytic lesion of the nucleus accumbens. ChAT was highly concentrated in the substriatal region, the neostriatum and the lateral part of the rostral substantia innominata. The strongest intensity of staining for acetylcholinesterase was found in the substriatal grey and the neostriatum. Very high GAD activity was found in the substantia innominata, being even slightly higher than that in the pars reticulata of the substantia nigra. The lateral preoptic area, the bed nucleus of the stria terminalis and the globus pallidus also showed high activity of GAD. After lesions of the nucleus accumbens the activity of GAD decreased significantly in the substantia innominata and in a restricted part of the rostroventral globus pallidus, but not in the other regions studied. ChAT activity and acetylcholinesterase staining were unaffected in all regions.

The results indicate that a dense GABAergic projection originates in the nucleus accumbens and terminates in the rostral substantia innominata and rostroventral part of the globus pallidus. The study gives neurochemical support to the suggestion that nucleus accumbens may be regarded as a ventral part of the neostriatum and that the rostral substantia innominata may be regarded as a ventral part of the globus pallidus.

INTRODUCTION

The basal forebrain of the rat is composed of several indistinctly outlined cell groups, among them the preoptic nuclei, substantia innominata and the bed nucleus of stria terminalis. However, despite the close proximity of these nuclei and similarities

in cytoarchitectonics, they receive afferent projections from different parts of the brain. The bed nucleus of stria terminalis is innervated predominantly by projections from the amygdala and subiculum[6,32], while the preoptic areas receive their major innervation from the septum and from the bed nucleus of stria terminalis[30]. The substantia innominata is especially difficult to distinguish from the lateral preoptic area[3,15,16,30], but recent work has shown that the rostral part of the substantia innominata as defined by Swanson[30] receives a dense projection from the nucleus accumbens septi[16,36] and from the olfactory tubercle[15]. The accumbens projection, which seems to continue into a restricted part of the rostroventral globus pallidus[31,36] is not shared by the other basal forebrain nuclei, and Heimer and co-workers have therefore introduced the terms 'ventral striatopallidal pathway' and 'ventral pallidum' to describe these fibres and their termination[15,16].

We have previously found a high activity of the γ-aminobutyric acid (GABA) marker enzyme, glutamate decarboxylase (GAD, EC 4.1.1.15), and an intermediate activity of the acetylcholine (ACh) marker enzyme, choline acetyltransferase (ChAT, EC 2.3.1.6), in what was tentatively described as substantia innominata and which should correspond to the 'ventral pallidum'[12]. In the present communication these observations have been extended. The detailed distribution of ChAT and GAD and the acetylcholinesterase (AChE, EC 3.1.1.7) staining pattern in the cell groups of the basal forebrain have been studied, special attention being paid to precise dissection. In particular, we have investigated whether the concept of 'ventral pallidum' with a 'ventral striatopallidal' projection is supported by neurochemical data. The transmitter in the 'ventral striatopallidal' fibres has also been identified. A preliminary report has been presented[35].

EXPERIMENTAL PROCEDURES

Surgical operations

Male Wistar rats of 170–200 g body weight, were anaesthetized with a mixture of diazepam and Hypnorm Vet 'Mekos', and the animals were placed in a Kopf stereotactic apparatus according to the atlas of König & Klippel[22]. A tungsten electrode (0.5 mm diameter), insulated to the tip, was lowered through a burr hole into the nucleus accumbens at coordinates A: 9.5, L: 1.0, V: −1.0, another electrode was clipped to the edge of the wound, and lesions were made with an alternating current (0.2 A) for 3–10 sec. Three sham-operated animals were subjected to the same procedure, but no current was applied. (All animals were operated on and/or sacrificed between 15 April and 15 May).

Tissue preparation

Five to seven days after operation, both lesioned and sham-operated rats, together with nine unoperated animals were decapitated between 09.00 and 11.00 h. The brains were rapidly removed and frozen with a CO_2 jet, and frontal 40 μm sections cut in a cryostat as previously described[11]. Sections from the whole rostrocaudal extent of the nucleus accumbens were thawed on microscope slides, air dried and

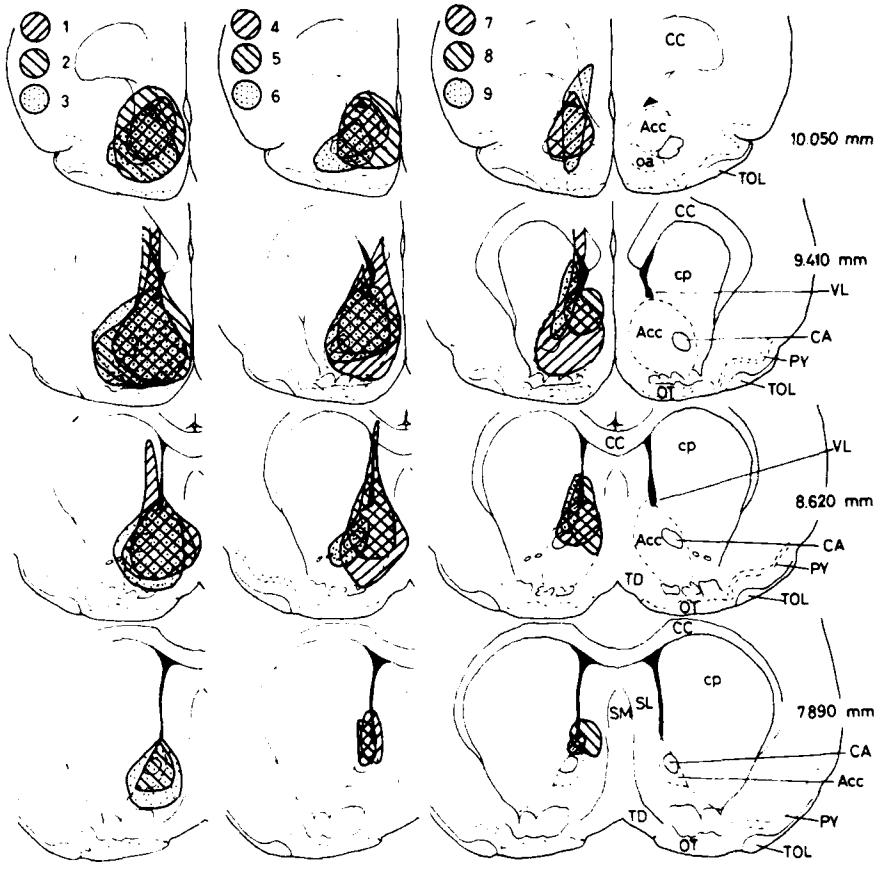

Fig. 1. Composite drawings showing the extent of the electrolytic lesions of nucleus accumbens in rats R1–R9. Acc, nucleus accumbens; CA, commissura anterior; CC, corpus callosum; cp, caudate-putamen; oa, anterior olfactory nucleus; OT, olfactory tubercle; PY, pyriform cortex; SL, lateral septum; SM, medial septum; TD, diagonal band; TOL, lateral olfactory tract; VL, lateral ventricle. Numbers indicate distance in front of interaural line. Modified from König and Klippel[22].

stained with thionin for mapping of the lesions (Fig. 1). Beginning at level A 7500[22], sections including the lateral preoptic area, globus pallidus or substantia nigra were either collected, freeze-dried and stored as previously described[11], or thawed on microscope slides and stained for AChE[29], or with routine cells stains (thionin or May Grünwald Giemsa).

Dissection

The regions to be studied were dissected under a binocular microscope which allowed corresponding stained and freeze-dried sections to be viewed simultaneously. Based on the morphological characteristics in our stained sections (Fig. 2) the following regions were taken out: a sample from the neostriatum immediately dorsal

328

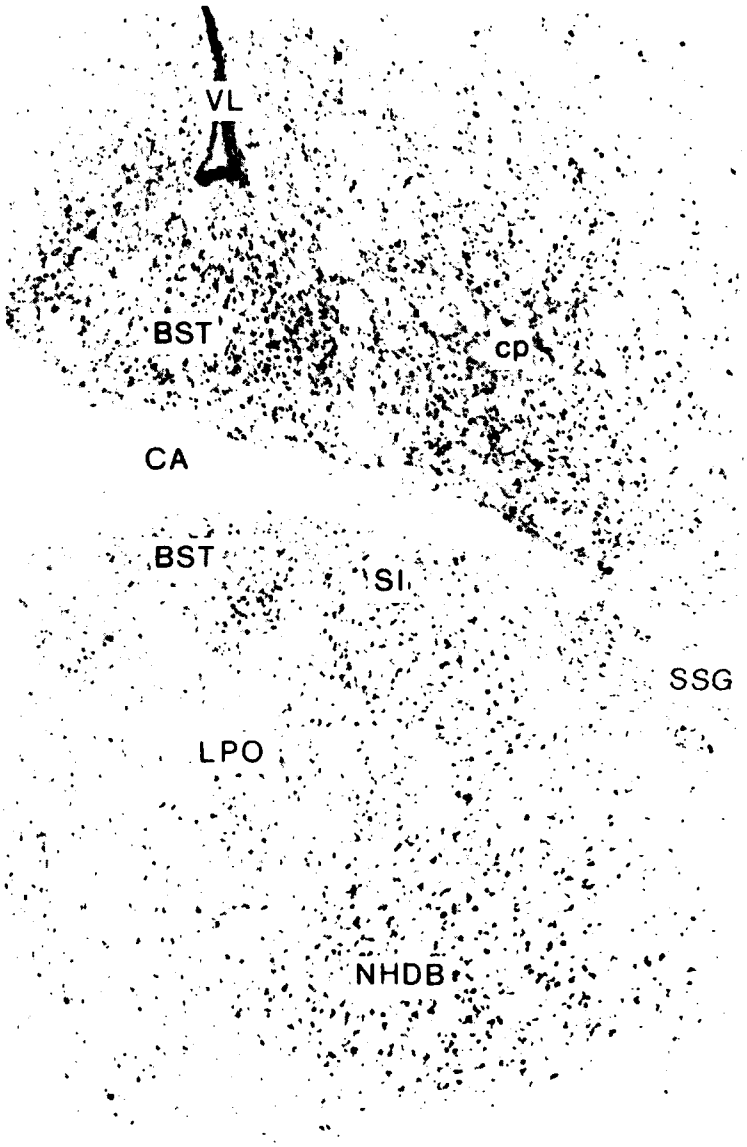

Fig. 2. Thionin stained frontal section (40 μm) of basal forebrain regions in the rat, taken from level A 7100[22]. BST, bed nucleus of the stria terminalis; LPO, lateral preoptic area; NHDB, nucleus of the horizontal limb of the diagonal band; SI, substantia innominata; SSG, substriatal grey region. Other abbreviations as in Fig. 1. × 30.

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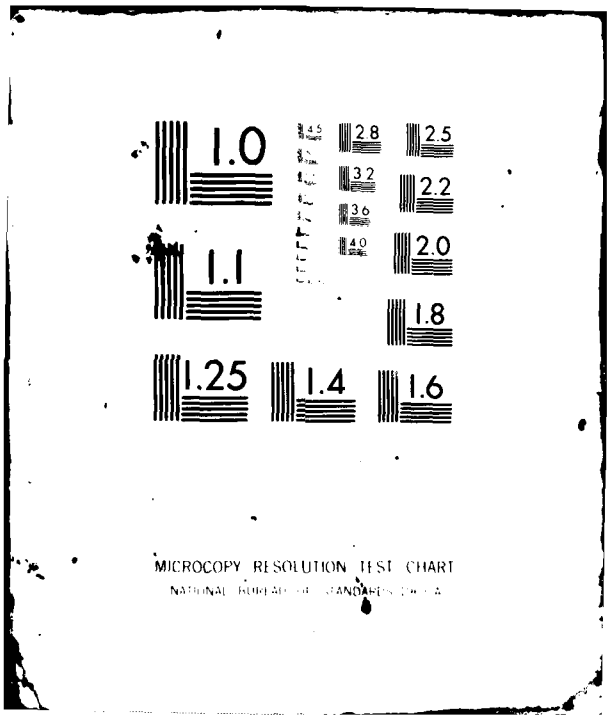
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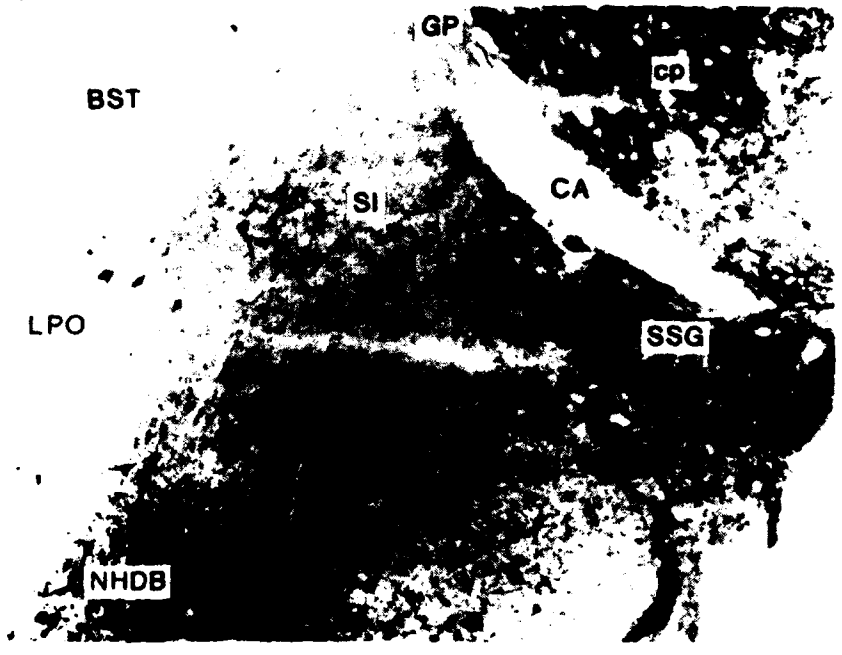

Fig. 3. AChE-stained frontal section (40 μm) of basal forebrain regions in the rat, taken from level A 6900[22]. GP, globus pallidus. Other abbreviations as in Figs. 1 and 2. × 37.

to the commissura anterior and extending from the bed nucleus of the stria terminalis medially to the medial border of the 'substriatal grey region' laterally, the bed nucleus of the stria terminalis both ventral and dorsal to the commissure[30], the lateral preoptic area immediately ventral to the bed nucleus, the substriatal grey region, and the rostral substantia innominata lying between the last two regions (Fig. 2). In unoperated animals the last sample was divided into lateral and medial parts. At more caudal levels, the rostroventral tip of the globus pallidus adjacent to the bed nucleus was dissected instead of the neostriatum. In addition, more caudal samples from the central part of globus pallidus and from the rostromedial pars reticulata of substantia nigra were taken for comparison.

Biochemical analysis

The samples (1-5 μg dry weight) were weighed on a fishpole quartz fibre balance[23], and assayed for ChAT or GAD activity with previously reported radiochemical methods[9,11,12]. Statistical significance between results was assessed with the non-parametric Wilcoxon two-sample test[17].

RESULTS

Histological staining of the basal forebrain (Fig. 2) revealed several neuronal

330

TABLE I

Distribution of choline acetyltransferase and glutamate decarboxylase in the basal forebrain and related regions of the rat

Results from 3–9 animals, presented as mean ± S.E.M. (number of samples).

Region	*Choline acetyl transferase*	*Glutamate decarboxylase*
	(μmol/h/g dry wt.)	
Neostriatum	84.3 ± 9.7 (4)	200 ± 22 (6)
Globus pallidus,		
rostroventral part	20.9 ± 3.2 (4)	700 ± 29 (6)
central part	—	546 ± 11 (5)
Bed nucleus of stria terminalis,		
ventral part	27.8 ± 1.3 (5)	619 ± 63 (7)
dorsal part	21.9 ± 2.2 (5)	446 ± 50 (7)
Lateral preoptic area	32.2 ± 3.3 (4)	755 ± 75 (5)
Substantia innominata,		
medial part	34.8 ± 4.3 (4)	1075 ± 71 (6)
lateral part	79.7 ± 8.5 (5)	957 ± 106 (8)
Substriatal grey region	150.0 ± 7.9 (5)	291 ± 43 (5)
Substantia nigra, pars reticulata	—	892 ± 70 (23)

populations showing different characteristics under optical microscopy. The ventral and dorsal parts of the bed nucleus of the stria terminalis both consisted of rather small, densely packed neurones. Ventral to this, the lateral preoptic area contained larger, more scattered cells, while the substantia innominata displayed irregular, large and unevenly scattered neurones (Fig. 2). Lateral to the substantia innominata and ventral to the temporal limb of the anterior commissure, the substriatal grey region contained small neurones, which resembled those of the neostriatum. The rostroventral part of globus pallidus, dissected at a slightly more caudal level, had similar cell morphology as substantia innominata. AChE stained sections (Fig. 3) demonstrated a dense staining in the neostriatum, the substriatal grey and the horizontal nucleus of the diagonal band. In contrast, the bed nucleus of the stria terminalis was almost devoid of staining product, while the globus pallidus and the lateral preoptic area had a few AChE positive neurones.

The substantia innominata had both some neuropil staining and AChE-positive neurons (Fig. 3). The latter are probably a dorsal extension of the dense aggregations of such cells in the horizontal diagonal band nucleus[8,18]. The distribution of ChAT in these regions (Table I) paralleled, with few exceptions, the AChE pattern. High activity was found in the substriatal grey and the caudate-putamen (neostriatum), while the bed nucleus and rostral globus pallidus displayed low activities. The lateral preoptic area and medial part of the substantia innominata had slightly higher ChAT activity than the bed nucleus, while the lateral substantia innominata had approximately the same activity as the caudate-putamen.

The GAD activity was distributed differently. Both parts of the substantia innominata showed very high activity. These were clearly higher than any parts of the

TABLE II

Glutamate decarboxylase in substantia innominata and globus pallidus following electrocoagulation of nucleus accumbens

Results presented as mean ± S.E.M. (number of samples). Lesioned side significantly lower than unlesioned side: **** $P < 0.001$; *** $P < 0.01$; ** $P < 0.02$; * $P < 0.05$ (Wilcoxon two-sample test).

Animal	*Substantia innominata*		*Rostroventral globus pallidus*		*Central globus pallidus*	
	Unlesioned side (μmol/h/g dry wt.)	*Lesioned side % of unlesioned*	*Unlesioned side (μmol/h/g dry wt.)*	*Lesioned side % of unlesioned*	*Unlesioned side (μmol/h/g dry wt.)*	*Lesioned side % of unlesioned*
R1	—	—	845 ± 24(6)	52 ± 4(6)****	582 ± 7 (3)	85 ± 18(3)
R2	1192 ± 73 (8)	29 ± 6(6)****	—	—	—	—
R3	841 ± 82 (3)	40 ± 7(3)*	540 ± 47(3)	60 ± 9 (4)*	—	—
R4	1099 ± 120(4)	49 ± 6(5)**	694 ± 44(5)	62 ± 5 (5)***	579 ± 14(3)	94 ± 4 (3)
R5	1002 ± 40 (6)	56 ± 4(6)****	596 ± 51(4)	65 ± 4 (4)**	490 ± 16(3)	99 ± 10(3)
R6	1361 ± 58 (11)	63 ± 5(11)****	859 ± 56(3)	82 ± 4 (3)*	983 ± 82(3)	82 ± 8 (3)
R7	1137 ± 108(9)	73 ± 5(10)***	683 ± 37(2)	87 ± 7 (4)*	—	—
R8	946 ± 54 (7)	99 ± 4(7)	654 ± 78(3)	100 ± 15(3)	658 ± 9 (3)	108 ± 8 (3)
R9	930 ± 147(5)	96 ± 9(7)	—	—	—	—

globus pallidus and even slightly higher than the activity in pars reticulata of substantia nigra (Table I). The rostroventral part of globus pallidus, the ventral part of the bed nucleus and the lateral preoptic area also showed high GAD activity, while in the dorsal part of the bed nucleus it was slightly less. Compared with these regions, the substriatal grey and the caudate-putamen showed rather low GAD activity.

Electrocoagulations of the nucleus accumbens (Fig. 1) caused different types of lesions depending on the duration of the lesioning current. In animals R1–R4 almost all accumbens tissue was destroyed, in R5–R7 the rostrodorsal part of the nucleus was destroyed but the ventrocaudal part was intact, and in R8 and R9 only a small part situated rostrodorsally was affected. In addition, the anterior olfactory nucleus, parts of the septum, and the medial caudate-putamen were affected to a limited degree in all animals (Fig. 1).

In lesioned animals the GAD activity on the contralateral, unlesioned side was similar to the activity in samples from unlesioned animals (compare Table I and II). The effect of lesion is therefore expressed as the percentage of the activity in the contralateral side of the lesioned animals (Table II).

Following lesions in rats R1–R7 the GAD activity in both substantia innominata and the rostroventral part of the globus pallidus decreased significantly. The maximum decrease was found after the largest accumbens lesions (Table II, R1–R4), while no significant decrease was found after the small lesions in R8 and R9. In contrast, there was no correlation between the extent of the septal or striatal involvement in the lesions and the decrease in GAD activity (compare the septal lesions in R1 vs R8 and the striatal lesions in R5 vs R9). GAD in the central part of the globus pallidus was unaffected by all types of lesions, and this was also true in the bed

TABLE III

Choline acetyltransferase in substantia innominata, rostral globus pallidus and ventral neostriatum following electrocoagulation of nucleus accumbens

Results from 6–15 samples from animals R3, R5, R6 and R7, presented as mean ± S.E.M.

	Unlesioned side (μmol/h/g dry wt.)	*Lesioned side % of unlesioned*
Substantia innominata	54.9 ± 5.6	106 ± 11
Rostroventral globus pallidus	21.0 ± 4.2	112 ± 8
Ventral neostriatum	83.3 ± 12.0	97 ± 11

nucleus of stria terminalis (94 ± 8%), lateral preoptic area (120 ± 14%) and in the neostriatum adjacent to the substantia innominata (105 ± 13%) (mean ±S.E.M., data from R1, R3, R4 and R5).

In contrast to GAD, ChAT activity in animals with large, intermediate or small accumbens lesions was unchanged in both the substantia innominata and the rostroventral globus pallidus (Table III). In addition, ChAT in samples from the neostriatum adjacent to the lesion was not changed (Table III), a finding which eliminates the possibility that an unspecific heat effect was responsible for the decrease in GAD activity in substantia innominata and globus pallidus. The AChE staining pattern was not visibly affected by any of the lesions.

DISCUSSION

Two points deserve special consideration in the present study: the identity of the transmitter in a major efferent projection of the nucleus accumbens, and the classification of the region in the basal forebrain where these fibres terminate.

The indistinct morphology of the basal forebrain in the rat has been an obstacle to a clear functional classification of the nuclei present[3,5,15,30]. This is also reflected in neurochemical studies, which have shown high concentrations of several putative transmitters in this region, but which — except for the bed nucleus of the stria terminalis — have failed to provide for a detailed neurochemical characterization of the different neuronal populations present[1,2,21,26]. In particular, the precise distribution of the very high GAD activity in the region has not been unequivocally defined[12,14,33]. In an effort to clarify these issues, we have in the present work characterized the morphology of the basal forebrain in the rat with thionin and AChE staining. The different nuclei were identified following the description of Swanson[30], and the activity of the specific ACh and GABA markers, ChAT and GAD[10], were analysed.

The results indicate that the neurones in the basal forebrain adjacent to the anterior commissure can be divided into 4 principal groups, e.g. the bed nucleus of the stria terminalis, the lateral preoptic area, the substantia innominata together with the

rostroventral globus pallidus, and the substriatal grey region together with the neostriatum. Morphologically, 3 regions are well discernible. The neurones in the lateral preoptic region and rostral substantia innominata are rather similar, and no clear border separates these nuclei. In contrast, the neurones in the bed nucleus of the stria terminalis are clearly different from those in the lateral preoptic area and the substantia innominata, and these regions are well separated by a cell-free zone. Similarly, the neurones in the substriatal grey region is markedly different from those in the substantia innominata, and a sharp border also separates these regions (Fig. 2). The rostroventral globus pallidus demonstrates morphological similarities with the substantia innominata and lateral preoptic area, while the neostriatum is similar to the substriatal grey region.

This classification is also, at least partly, reflected in the transmitter composition of the regions. The bed nucleus of the stria terminalis has a dense population of GABAergic fibres, but an unimportant cholinergic innervation, in agreement with other reports[1,2]. In contrast, the substriatal grey region is densely innervated by cholinergic fibres, and has only a sparse population of GABAergic terminals, like the neostriatum. The lateral preoptic area, the rostral substantia innominata and the rostroventral globus pallidus seem to be intermediate. They all have a population of AChE-positive cells, which are most numerous in the substantia innominata. The cholinergic innervation of these nuclei as indicated by the ChAT activity is quantitatively unimportant in the rostroventral globus pallidus, more dense in the lateral preoptic area, and quite considerable in the lateral substantia innominata. In contrast, a very dense GABAergic innervation is found in all these nuclei. These GABA terminals are most highly concentrated in the rostral substantia innominata, which has even higher GAD activity than the pars reticulata of the substantia nigra.

Thus, only small morphological and neurochemical differences are found between the lateral preoptic area and the rostral substantia innominata and rostroventral globus pallidus. The 3 regions differ, however, in their fibre connections. The lateral preoptic area receives fibres from the septum and sends efferents to the habenula[15,24,30], while the rostral substantia innominata and the rostroventral globus pallidus have a major input from the nucleus accumbens and do not project fibres to the habenula[15]. Also, the origin of the GABAergic fibres in the regions seems to be different. In the present work, the lesion experiments indicate that most of the GABA terminals in the rostral substantia innominata and the rostroventral part of globus pallidus originate in the nucleus accumbens while those in the lateral preoptic area do not. Thus, the decrease in GAD in the substantia innominata and rostroventral pallidum correlated well with the extent of the accumbens lesion, it was found to be independent of the involvement of the septum or the neostriatum, and it was restricted to those parts of the basal forebrain and globus pallidus which are known to receive accumbens fibres. These results make it unlikely that the decrease in GAD activity was the result of damage to fibres passing through the nucleus accumbens but originating elsewhere. Judged on the amount of GAD activity destroyed by this lesion, this fibre tract must be one of the most dense GABA projections in the rat brain.

We thus suggest that most of the GABA terminals in the rostroventral globus

pallidus and the rostral substantia innominata belong to an accumbens-related functional system[28], which is clearly different from that of the hypothalamus-related preoptic nuclei[5,30]. The nucleus accumbens has traditionally been regarded as part of the 'limbic system'[16,27]. However, recent anatomical and biochemical data indicate that the nucleus in many ways is similar to the neostriatum, both regarding afferent connections and intrinsic neurones[12,16,34]. It is therefore interesting that the present work also indicates similarities in efferent connections. Thus, the neostriatum has been found to send a dense GABAergic projection to the main body of globus pallidus[13,19], whereas nucleus accumbens, as shown in the present work, probably sends a GABAergic projection to a ventral extension of this nucleus. This finding consequently gives additional credence to the proposal that the nucleus accumbens might be regarded as a 'ventral striatum', that the rostral substantia innominata might be regarded as a 'ventral pallidum', and that these two regions are connected by a 'ventral striatopallidal' projection[15,16]. The remainder of the GAD activity in the 'ventral pallidum' may possibly derive from fibres originating in the olfactory tubercle. Although these fibres, which probably would be unharmed by our lesions (Fig. 1), terminate in a slightly more ventral and lateral part of the substantia innominata than the fibres from the nucleus accumbens[15], they would still be included in our samples.

The origin of cholinergic fibres in the basal forebrain remains unanswered by this study. However, we have previously shown that the basal forebrain does not receive ascending cholinergic fibres[12], and it has also been shown that the ChAT activity in samples from the lateral preoptic area which probably included the substantia innominata as defined in this paper, is decreased by local kainic acid application[8]. It is therefore possible that most of these cholinergic fibres are derived from local neurones, some of which probably belong to the predominantly cholinergic system of 'magnocellular nuclei of the basal forebrain', which project widely to the cortex and brain stem[7,20,25].

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BIOCHEMICAL EVIDENCE FOR γ-AMINOBUTYRATE CONTAINING FIBRES FROM THE NUCLEUS ACCUMBENS TO THE SUBSTANTIA NIGRA AND VENTRAL TEGMENTAL AREA IN THE RAT

I. WALAAS and F. FONNUM
Norwegian Defence Research Establishment, Division for Toxicology, N-2007 Kjeller, Norway

Abstract Glutamate decarboxylase activity, a specific marker for γ-aminobutyrate-containing neurons, has been analysed in microdissected samples from rat mesencephalon following unilateral electrocoagulations of the nucleus accumbens. This lesion resulted in a consistent decrease of 50% in the enzyme activity in the rostromedial substantia nigra, and a slight, but insignificant decrease (−15%) in the medial parts of the caudal pars compacta of the substantia nigra. No change was found in the lateral pars compacta or the central pars reticulata. In the ventral tegmental area, the highest activity was found in the rostromedial part, adjacent to the mammillary body. At this level, a significant decrease of 20% was found in the ventral tegmental area on the lesioned side. In contrast, the activities in the medial accessory optic nucleus and the caudal ventral tegmental area adjacent to the interpenduncular nucleus were unchanged

The results indicate that the nucleus accumbens sends γ-aminobutyrate-containing fibres to the rostromedial substantia nigra and to the rostral ventral tegmental area. The caudal ventral tegmental area, the lateral pars compacta and the central pars reticulata do not receive measurable amounts of such fibres.

THE NUCLEUS accumbens septi distributes nerve fibres to a considerable number of brain regions, and seems to be able to exert a widespread influence on brain functions (POWELL & LEMAN, 1976; CONRAD & PFAFF, 1976; WILLIAMS, CROSSMAN & SLATER, 1977; NAUTA, SMITH, FAULL & DOMESICK, 1978). One of the quantitatively dominating projections terminates in the mesencephalon, where the fibres distribute densely to rostral parts of the ventral tegmental area of Tsai and the dorsomedial substantia nigra (SWANSON & COWAN, 1975; CONRAD & PFAFF, 1976; NAUTA *et al.*, 1978; PHILLIPSON, 1978). These fibres may be important both as a regulator of the 'mesolimbic' dopamine neurons in the ventral tegmental area, which in turn project bact to the nucleus accumbens (UNGERSTEDT, 1971; SIMON, LEMOAL, GALEY & CARDO, 1976; NAUTA *et al.*, 1978), and as an input to the nigral complex. The identification of the neurotransmitter(s) in these fibres would therefore be of considerable interest. γ-Aminobutyric acid (GABA) has potent actions on the dopamine-containing cells in the ventral tegmental area (FUXE, HÖKFELT, LJUNGDAHL, AGNATI, JOHANSON & PÉREZ DE LA MORA, 1975; PALFREYMAN, HUOT, LIPPERT & SCHECHTER, 1978) and has been proposed as a transmitter candidate on physiological and pharmacological grounds (WOLF, OLPE, AVRITH & HAAS, 1978; STEVENS, 1979). However, previous neurochemical studies have given conflicting results (FONNUM, WALAAS & IVERSEN, 1977; MCGEER, MCGEER & HATTORI, 1977; WADDINGTON & CROSS, 1978). The present study was initiated in an effort to resolve this problem, taking advantage of the precise and restricted distribution of the fibres from the nucleus accumbens (NAUTA *et al.*, 1978). Following unilateral electrocoagulations of the nucleus accumbens we have dissected parts of the substantia nigra and ventral tegmental area from freeze-dried sections, and analysed the activity of the specific GABA marker glutamate decarboxylase (GAD, EC 4.1.1.15), on both the lesioned and unlesioned side. Adjacent regions have been analysed as control. Some preliminary results have been presented (WALAAS & FONNUM, 1979*a*).

Abbreviations: GABA, γ-aminobutyrate; GAD, L-glutamate decarboxylase.

EXPERIMENTAL PROCEDURES

Materials

White Wistar male rats of 180–210 g body weight were from Mollergaard-Hanssens Avlslab oratorium, Denmark. L-[1-^{14}C]glutamic acid was from The Radiochemical Centre, Amersham, U.K.

Lesions

The animals were anaesthetized with fentanyl citrate and fluanisone (Hypnorm vet., Mekos, 0.1 ml s.c.), and valium (Roche, 0.75 mg i.p.), and placed in a stereotactic frame after KÖNIG & KLIPPEL (1963). The skull was opened with a dental drill, and a tungsten electrode insulated to the tip (0.5 mm diameter) was lowered into the nucleus accumbens 9.5 mm in front of the interaural line, 1.2 mm lateral to the midline and 5.5 mm below the dura. Another electrode was clipped to the wound edge, and lesions were made with an alternating current (0.2 A) for 10 s. The lesions were placed randomly on the right or the left side in different rats.

Tissue preparation

Five six days after operation the rats were decapitated, the brains rapidly removed and frozen on a microtome

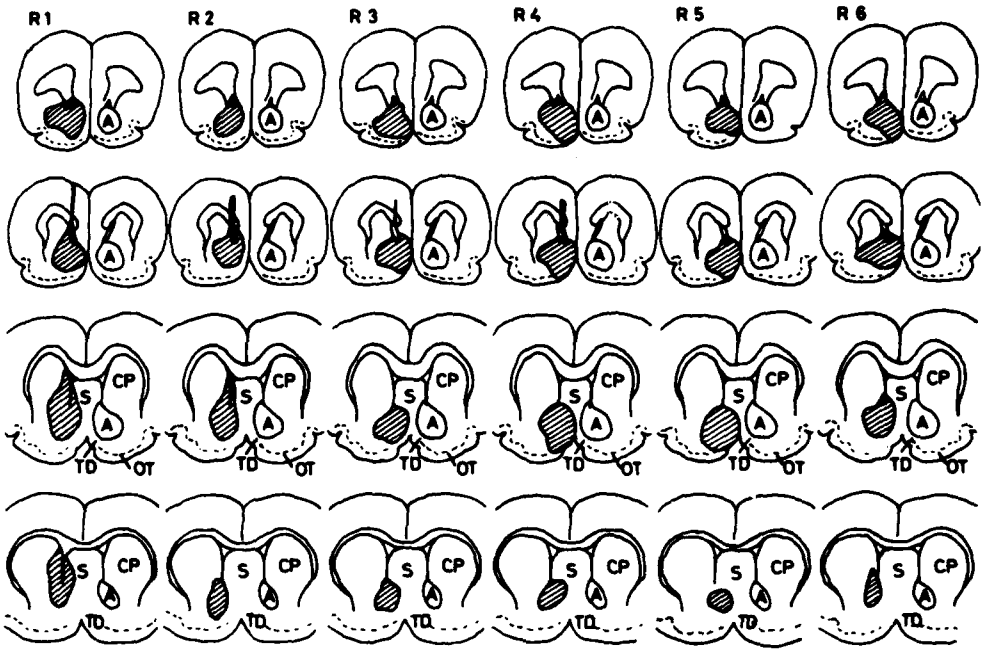

FIG. 1. Schematic outline of lesions destroying the nucleus accumbens in animals R1–R6. A, nucleus accumbens; CP, caudatoputamen; S, septum; OT, olfactory tubercle; TD, diagonal band nucleus.

chuck with a CO_2 jet. A knife mark was made in the cortex on the lesioned side, and frontal 40 μm sections were cut at −16 C. Sections from the rostral telencephalon were thawed on microscopic slides, air-dried and stained with thionin for mapping of the lesions (Fig. 1 and Table 1). Between levels A 2800 and A 1300 (KÖNIG & KLIPPEL, 1963) each 6th section was taken for cell-staining, while the intermediate sections were collected, freeze-dried and stored as previously described (FONNUM, STORM-MATHISEN & WALBERG, 1970).

Histological preparation

Unoperated animals were perfused through the ascending aorta with 50 ml isotonic saline followed by Bouin's fixative for 30 min. The brains were fixed overnight in Bouin's fixative, dehydrated, embedded in paraffin and sectioned in series at 5 μm. Every tenth section from the mesencephalon was mounted and stained with toluidine blue.

Dissection

The freeze-dried sections were inspected simultaneously with adjacent cell-stained 40 μm sections from the same animal in a stereomicroscope. The lesioned side was identified by means of the knife cut in the cortex, and the samples were dissected with broken razor-blade splints. Rostrally, the medial accessory optic nucleus, the fasciculus retroflexus and the mammillary penduncle were easily visible and used as reference points. The ventral tegmental area was dissected between the mammillary peduncle, the medial lemniscus and the medial accessory optic nucleus. The rostromedial substantia nigra was dissected adjacent to the medial accessory optic nucleus, and the central parts reticulata of the substantia nigra was dissected dorsal to the crus cerebri (Fig. 2). Some samples were also taken from the medial accessory optic nucleus. More caudally the ventral tegmental area was taken adjacent to the easily visible interpeduncular nucleus, while the substantia nigra was divided into medial and lateral pars compacta and central pars reticulata (Fig. 2).

Some of the dissected sections were mounted on egg albumin-glycerol coated slides, fixed in 10% formalin and stained for acetylcholinesterase (STORM-MATHISEN, 1970) in order to vizualize the ventral tegmental area and pars compacta of the substantia nigra (FONNUM *et al.*, 1977). Inspection of these slides revealed a precise dissection of

TABLE 1. EXTENT OF ELECTROCOAGULATIONS OF THE NUCLEUS ACCUMBENS

Animal	Nucleus accumbens	Caudatoputamen	Olfactory tubercle	Diagonal band nucleus	Bed nucleus of stria terminalis	Septum
R1	+ + + +	+(+)	–	–	+ + d	+
R2	+ + + +	+	–	–	–	–
R3	+ + + +	–	+ r	+ r	–	+ +
R4	+ + + +	–	+ +	+ +	–	+ +
R5	+ + + +	–	+ +	+ +	–	+ +
R6	+ + + +	+	–	+ r	+ d	+

r, Rostral; d, dorsal. The number of plus signs indicates the extent of destruction

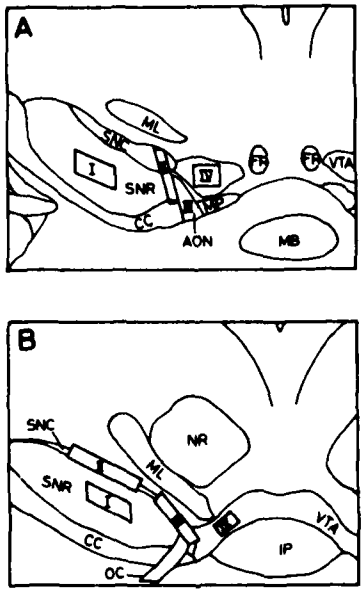

FIG. 2. Drawings based on toluidine-stained sections from the mesencephalon, demonstrating approximate outline of the different nuclei and localization of dissected samples. (A) rostral mesencephalon. Dissected samples: I, central pars reticulata; II, rostromedial substantia nigra; III, medial accessory optic nucleus; IV, ventral tegmental area. (B) Caudal mesencephalon. I, central pars reticulata; II, lateral pars compacta; III, medial pars compacta; IV, ventral tegmental area.

Abbreviations: AON, medial accessory optic nucleus; FR, fasciculus retroflexus; IP, interpeduncular nucleus; CC, crus cerebri; NR, nucleus ruber; ML, medial lemniscus; MB, mammillary body; MP, mammillary peduncle; SNC, substantia nigra, pars compacta; SNR, substantia nigra, pars reticulata; OC, oculomotor nerve.

the accessory optic nucleus, the ventral tegmental area and the rostromedial substantia nigra, while some samples from the caudal pars compacta extended slightly into the dorsal pars reticulata, or into the tissue between the substantia nigra and the medial lemniscus. Thus, some variation would be expected in the results from the caudal pars compacta, especially in the lateral part.

Biochemical analysis

Under microscopic guidance the samples (0.2–1.5 μg dry weight) were collected, moved to a fishpole quartz fibre balance (LOWRY, 1953), weighed and transferred to the assay tubes. Glutamate decarboxylase (GAD) activity was analysed by a CO_2-trapping method as described (FONNUM *et al.*, 1970; 1977), with a final concentration of 20 mM L-glutamic acid. The Wilcoxon two sample test was used to evaluate the results statistically.

RESULTS

Histological studies

Histological staining of sections from the mesencephalon was performed in order to characterize the neuronal population present in the regions investigated in this study (Fig. 2). In the rostral samples, the medial accessory optic nucleus was found between the crus cerebri and the mammillary peduncle. It extended from the ventral surface of the brain, between the medial substantia nigra and the ventral tegmental area, and the medium-sized cells found seemed to be preferentially oriented in a ventromedial dorsolateral direction (Fig. 3). This nucleus was easily visible in the freeze-dried sections, having a more dense appearance than adjacent regions when illuminated from below. The rostromedial nigral sample comprised a region extending from the dorsal border of the crus cerebri to the dorsal parts of the medial pars compacta. Medially, this sample bordered upon the accessory optic nucleus, and it extended laterally 0.1–0.2 mm into the substantia nigra (Fig. 2). Ventrally, this sample contained tissue from the pars reticulata, with a few medium-sized neurons, while dorsally a dense population of both fusiform and pyramidal pars compacta neurons (FALLON, RILEY & MOORE, 1978; JURASKA, WILSON & GROVES, 1977) was included. The ventral tegmental area comprised more scattered neurons, most of them being identical in appearance to the pars compacta cells (Fig. 3).

In more caudal regions, the medial pars compacta was pierced by fascicles from the oculomotor nerve and by perforating blood vessels. Laterally it blended with adjacent regions without any distinct border. Also at this level, the pars compacta neurons extended without interruption into the caudal ventral tegmental area, where they became more sparse in the region dorsolateral to the interpeduncular nucleus (Fig. 2).

Effects of the lesion on glutamate decarboxylase activity

Anatomical studies have demonstrated a strict unilateral distribution of both the striatonigral (BUNNEY & AGHAJANIAN, 1976; TULLOCH, ARBUTHNOTT & WRIGHT, 1978) and the accumbens mesencephalic fibres (NAUTA *et al.*, 1978), and we have previously shown that a hemitransection of the brain is without effect on GAD in the contralateral mesencephalon (FONNUM *et al.*, 1977). In the present study, we have therefore used the unlesioned side as 'normal', and compared the biochemical results found here with those on the lesioned side.

Distribution of glutamate decarboxylase in the mesencephalon. On the unlesioned side, the highest GAD activity was found in the central pars reticulata and in samples from the rostromedial substantia nigra (Table 2). The latter sample comprised both the pars reticulata and the pars compacta, and separate analysis of these regions revealed equal activities in both (results not shown). Lower activity was found in both medial and lateral parts of the caudal pars compacta (Table 3). In the ventral tegmental area, the highest activity was found rostrally (Tables 2 and 3). The distribution of GAD in the caudal ventral tegmental area and caudal pars compacta is in general agreement with our previous results (FONNUM *et al.*, 1977).

Table 3. Glutamate decarboxylase activity in mesencephalic nuclei following electrocoagulation of the nucleus accumbens

Region	Contralateral side to lesion (μmol h g dry wt)	Ipsilateral side to lesion (% of unlesioned)
Caudal ventral tegmental area	178 ± 15(9)	97 ± 9(15)
Caudal substanti nigra, medial pars compacta	354 ± 34(14)	85 ± 11(11)
Caudal substantia nigra, lateral pars compacta	396 ± 67(11)	98 ± 22(10)
Medial accessory optic nucleus	190 ± 12(9)	92 ± 5(8)

Results from five animals, presented as mean ± S.E.M. (number of samples).

The medial accessory optic nucleus had distinctly lower activity than the adjacent ventral tegmental samples, and very much lower than the rostromedial nigral region immediately lateral to it (Tables 2 and 3).

Extent of the lesions. Inspection of the telencephalic sections demonstrated that the nucleus accumbens as usually defined (König & Klippel, 1963) has been destroyed in all animals, and that the globus pallidus, the caudal caudatoputamen and the anterior hypothalamus were unharmed (Fig. 2). However, the lesions were not restricted to the nucleus accumbens. In animals R1, R2 and R6, a part of the rostromedial caudatoputamen was also involved, and in R1 and R6 the lesion also encroached slightly upon the lateral septum and rostral parts of the dorsal bed nucleus of the stria terminalis (Table 1). The nucleus of the diagonal band was involved in animal R6. In animals R3, R4 and R5 the lesion left the caudatoputamen and the bed nucleus of the stria terminalis intact. However, these lesions involved most of the ventral ipsilateral septum and the nucleus of the diagonal band. Animals R4 and R5 also had small lesions in the mediorostral olfactory tubercle (Table 1).

Glutamate decarboxylase ipsilateral to the lesion. Biochemical analysis on the lesioned side showed that the activity of GAD was markedly reduced in the rostromedial substantia nigra but not in the adjacent medial accessory optic nucleus. This decrease was found both in ventral and dorsal parts of this sample, and was independent of the involvement of the septum, the caudatoputamen, the nucleus of the diagonal band or the bed nucleus of the stria terminalis (Table 2). Therefore it seems to be a specific result of the accumbens lesion only. In the ventral tegmental area rostral to the interpenduncular nucleus, smaller decreases in GAD (10-31%) were found in five animals. These decreases were most substantial in animals R3 and R5, both with considerable septal involvement, but they were also statistically significant in R2 and R6 ($P < 0.05$ and $P < 0.01$, respectively), animals where the septum was barely touched by the lesion.

A slight but insignificant decrease in GAD activity was also found in the medial part of the caudal pars compacta of the substantia nigra. The lateral pars compacta, the central pars reticulata, the caudal ventral tegmental area and the medial accessory optic nucleus, regions which receive few or no fibres from the nucleus accumbens (Nauta *et al.*, 1978) all displayed unchanged activity after the lesions (Table 2).

DISCUSSION

γ-Aminobutyrate in the mesencephalon

The GABAergic fibres reaching the mesencephalon probably have an important influence on the local dopaminergic cells (Cheramy, Nieoullon & Glowinski, 1978; Cattabeni, Bugatti, Groppetti, Maggi, Parenti & Racagni, 1978), but recent studies have shown that they also may be involved in behaviour not mediated by monoamines (Scheel-Krüger, Arnt & Magelund, 1977; Di Chiara, Porceddi, Morelli, Mulas & Gessa, 1978; Waddington, 1978; see also review by Dray, 1979). Thus, the functional organization of these fibres may be more complicated than previously believed. The major GABAergic input to these regions has been well defined: it arises in the caudatoputamen and possibly in the globus pallidus and terminates in the substantia nigra (Kim, Bak, Hassler & Okada, 1971; Hattori, McGeer, Fibiger & McGeer, 1973; Fonnum, Grofová, Rinvik, Storm-Mathisen & Walberg, 1974; Brownstein, Mroz, Tappaz & Leeman, 1977; Fonnum, Gottesfeld & Grofová, 1978; Jessell, Emson, Paxinos & Cuello, 1978). However, other minor GABAergic projections may also terminate parts of this nucleus, especially in the pars compacta (Fonnum *et al.*, 1977; Brownstein *et al.*, 1977). In addition the GABAergic innervation to the medialmost mesencephalic nuclei, which also might be functionally important (Stevens, 1979) is as yet undefined; the present study shows that these regions have a highly specific pattern of GABAergic innervation. The highest GAD activities in the pars compacta of the substantia nigra and the ventral tegmental area were found in the rostral mesencephalon. At this level the rostromedial pars compacta and pars reticulata exhibited the same density of GABAergic terminals as the central pars reticulata, while the caudal pars compacta had only 60% of this GAD activity. Considerably lower activities were found in the medial accessory optic nucleus, the caudal ventral tegmental area and the interpeduncular nucleus (Fonnum *et al.*, 1977, and this paper). These data lead us to suggest that, at least in the rat, both the medial part of the dopaminergic cells group

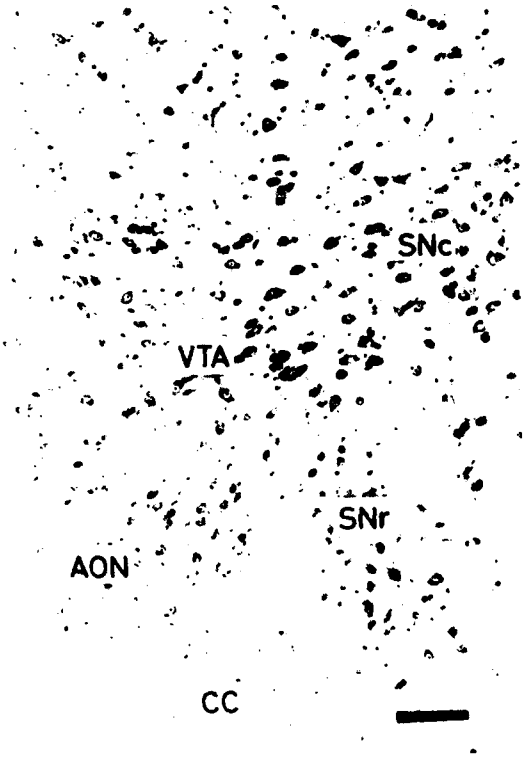

FIG. 3. Toluidine-stained section showing the cytological features of the rostromedial substantia nigra and adjacent regions. Compare with Fig. 2. Abbreviations as in Fig. 2. Bar: 100 μm.

TABLE 2. GLUTAMATE DECARBOXYLASE ACTIVITY IN THE SUBSTANTIA NIGRA AND THE VENTRAL TEGMENTAL AREA FOLLOWING THE DEGENERATION OF THE NUCLEUS ACCUMBENS

	Rostral ventral tegmental area		Rostromedial substantia nigra		Substantia nigra, central pars reticulata	
Animal	Contralateral side to lesion (μmol h g dry wt)	Ipsilateral side to lesion (% unlesioned)	Contralateral side to lesion (μmol h g dry wt)	Ipsilateral side to lesion (% unlesioned)	Contralateral side to lesion (μmol h g dry wt)	Ipsilateral side to lesion (% unlesioned)
R1	190 ± 41 (3)	85 ± 6 (4)	593 ± 76 (3)	31 ± 14 (3)	544 ± 14 (5)	87 ± 25 (3)
R2	271 ± 40 (9)	83 ± 5 (5)	476 ± 62 (8)	49 ± 4 (8)	490 ± 14 (5)	99 ± 4 (5)
R3	330 ± 20 (8)	69 ± 5 (10)	661 ± 72 (6)	51 ± 5 (9)	900 ± 98 (3)	81 ± 25 (3)
R4			543 ± 70 (11)	50 ± 6 (12)	747 ± 79 (3)	81 ± 9 (3)
R5	263 ± 21 (8)	77 ± 4 (9)	766 ± 32 (3)	65 ± 6 (3)	695 ± 32 (3)	130 ± 18 (3)
R6	250 ± 21 (5)	91 ± 10 (5)	415 ± 46 (6)	71 ± 4 (5)	758 ± 85 (6)	101 ± 5 (7)
Pooled data	277 ± 15 (33)	79 ± 3 (33)*	545 ± 62 (36)	51 ± 6 (40)**	658 ± 51 (25)	99 ± 12 (24)

Results presented as mean ± S.E.M. (number of samples). **$P < 0.001$. *$P = 0.001$ (Wilcoxon two sample test).

A9 in the pars compacta, and the dopaminergic cell group A10 in the ventral tegmental area (Dahlström & Fuxe, 1965) might preferentially be innervated by GABAergic fibres rostrally.

Afferents from the nucleus accumbens. Our study also defines the origin of one of the GABAergic inputs to these regions: a significant population of GABAergic terminals disppears from both the rostromedial substantia nigra, the rostral ventral tegmental area and possibly a part of the caudomedial pars compacta after destruction of the nucleus accumbens. This effect is not dependent on the involvement of nuclei adjacent to the nucleus accumbens, and the distribution of these GABAergic terminals coincides well with the termination of the efferent fibres from the nucleus accumbens as visualized by anatomical methods (Conrad & Pfaff, 1976; Nauta *et al.*, 1978). Thus, these GABAergic fibres probably originate in the nucleus accumbens proper and do not represent fibres *en passage* destroyed by the lesion. Some fibres from the nucleus accumbens also extend into the lateral pars compacta (Nauta *et al.*, 1978), and these might also possibly contain small contingents of GABAergic fibres. If so however, they are too few to be detected by the techniques employed in the present study. In contrast to these regions we find that the caudal A10 group in the ventral tegmental area is not innervated by measurable amounts of GABAergic fibres from the nucleus accumbens. This is in agreement with our previous studies on hemitransected brains (Fonnum *et al.*, 1978). The GABA system in the medial accessory optic nucleus is also unaffected by the lesion, and the latter results are both in agreement with the anatomical studies since a small number if any of the accumbens fibres terminate in these regions (Nauta *et al.*, 1978). Our study also shows that the GABAergic fibres from the nucleus accumbens can only be found with precise microdissection techniques. This underlines that the negative findings on GAD in samples of whole substantia nigra after large lesions in the anterior neostriatum (Nagy, Carter & Fibiger, 1978) may not be entirely correct.

The present study further demonstrates that the nucleus accumbens in many ways behaves like an integral part of the rostroventral neostriatum. Firstly, *the biochemical identification of GABA as a transmitter* in the accumbens nigral pathway is consistent with the findings for the striatonigral projection (Fonnum *et al.*, 1974; Fonnum *et al.*, 1978). Secondly, the rostromedial termination of these GABAergic fibres in the substantia nigra is comparable to the distribution of the rostral neostriatal fibres, which are known to terminate preferentially in medial parts of the substantia nigra, while the caudal neostriatum sends fibres more laterally (Bunney & Aghajanian, 1976; Tulloch *et al.*, 1978). The dorsal termination of the accumbens fibres also agrees with anatomical studies, which have shown that the ventral neostriatum sends fibres preferentially to dorsal nigral regions, while the dorsal neostriatum sends fibres to the ventral nigra (Domesick, 1977; Nauta *et al.*, 1978). Lastly, our quantitative results show that the majority of the GABAergic fibres from the nucleus accumbens to the mesencephalon terminates in the medial substantia nigra and not in the ventral tegmental area. They therefore probably innervate cells which are situated laterally to those dopaminergic cells in the ventral tegmental area which project back to the nucleus accumbens. This projection pattern has also been found in the neostriatonigral pathway (Domesick, 1977; Fallon & Moore, 1978): restricted parts of the neostriatum project most densely to nigral regions immediately lateral to those nigral cells which innervate that particular striatal locus. Taken together, these data give additional credence to the proposed neostriatal nature of the nucleus accumbens (Heimer & Wilson, 1975; Swanson & Cowan, 1975; Nauta *et al.*, 1978; Nauta, 1979; Walaas & Fonnum, 1979*b*.

Possible functional implications

The nigral neurons influenced by the GABAergic fibres from the nucleus accumbens have not been defined (Nauta *et al.*, 1978). It has been shown that GABAergic terminals in the rostral substantia nigra influence nigrostriatal dopaminergic cells (James & Starr, 1978) and the accumbens fibres could well be involved in this system. However, also nigrotectal, nigrothalamic or nigroreticular neurons (Carpenter, Nakano & Kim, 1976; Rinvik, Grofová & Ottersen, 1976; Graybiel, 1978; Beckstead, Domesick & Nauta, 1979) in the pars reticulata may be involved. It is well known that locomotor activity may be initiated by dopaminergic mechanisms in the nucleus accumbens (Pijnenburg & Van Rossum, 1973), and the lack of this dopamine effect has indeed been suggested to be responsible for the akinesia found in the Parkinsonian syndrome (Andén & Johnels, 1977). The GABAergic projection from the nucleus accumbens to the substantia nigra, or alternatively the very dense GABAergic projection to the ventral pallidum (Walaas & Fonnum, 1979*b*) could possibly represent output pathways mediating these motor responses, as both these nuclei seem to have connections to the central motor system via the thalamus or the reticular substance (Heimer, 1978; Carpenter *et al.*, 1976; Beckstead *et al.*, 1979).

Lastly, our results demonstrate that some GABAergic fibres pass from the nucleus accumbens to the rostromedial part of the ventral tegmental area. Retrograde transport techniques and electrophysiological studies indicate that this region contains only few cells projecting to the nucleus accumbens (Nauta *et al.*, 1978; Woolf *et al.*, 1978), and our results therefore do not seem to give strong evidence in support of a mesolimbic inhibitory 'feed-back' system, i.e. that dopaminergic A10 cells are influenced by a GABAergic projection from the nucleus accumbens. The majority of the mesolimbic dopaminergic cells projecting to the nucleus accumbens, as demonstrated

by retrograde transport techniques, is located more caudally (Nauta *et al.*, 1978), and these cells are not innervated by GABAergic fibres from the rostral forebrain (Fonnum *et al.*, 1977; and this paper).

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